

APPENDIX

RESEARCH ON NAVY-RELATED COMBAT CASUALTY CARE ISSUES, NAVY OPERATIONAL- RELATED INJURIES AND ILLNESSES AND APPROACHES TO ENHANCE NAVY/MARINE CORPS PERSONNEL COMBAT PERFORMANCE

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ANALYSIS OF VOLATILE CONTAMINANTS IN U.S. NAVY FLEET SODA LIME

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13. ABSTRACT (Maximum 200 words) <p>Contamination was suspected of U.S. Navy Fleet soda lime (High Performance Sodasorb®) when an ammonia-like odor was reported during its use in August 1992. This material contained indicator dye and was used for carbon dioxide absorption during diving. This incident had a major impact on the U.S. Navy diving program when the Navy temporarily banned use of Sodasorb® and authorized Sofnolime® as an interim replacement. The Naval Medical Research Institute was immediately assigned to investigate. Testing involved sampling from the headspace (gas space) inside closed buckets and from an apparatus simulating conditions during operational diving. Volatile organic compounds were analyzed by gas chromatography and mass spectrometry; ammonia and amines were measured by infrared spectroscopy. Significant amounts of ammonia (up to 30 ppm), ethyl and diethyl amines (up to several ppm), and various aliphatic hydrocarbons (up to 60 ppm) were detected during testing of both Sodasorb® and Sofnolime®. Contaminants were slowly removed by gas flow and did not return. The source(s) of the ammonia and amines are unknown, although they may result from the breakdown of the indicator dye. Hydrocarbon contamination appeared to result from the materials of which the bucket is constructed. Based on these findings, the U.S. Navy is expected to phase in non-indicating soda lime that will be required to meet defined contaminant limits. This report presents: 1) in-depth description of procedures used during the investigation for contaminant analysis and 2) detailed results from all samples tested.</p>						
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INTRODUCTION

Soda lime is widely used as a carbon dioxide (CO_2) absorbent in underwater breathing systems and hyperbaric facilities. For the past few years, the U.S. Navy has purchased most of its soda lime for diving from one manufacturer (W.R. Grace & Co., Lexington, MA). This soda lime was loosely packaged in high-density polyethylene, 5-gallon, round buckets with a net weight of 37 lb. The absorbent (High Performance Sodasorb[®]) contained the indicator dye, ethyl violet, to allow users to monitor by color change the decline in CO_2 absorbent capacity of the soda lime. Anecdotal information suggests that the amount of indicator dye in Fleet Sodasorb[®] has increased over time, which is confirmed by a recent report from the manufacturer (1). Unfortunately, the latest Department of Defense specification on soda lime (2), written in 1953, is not applicable to present requirements and practices, including packaging and type of indicator dye. For these reasons, soda lime for diving use has been procured for a number of years by the U.S. Navy without a detailed military specification and, thus, without defined levels for indicator dye.

Contamination of U.S. Navy Fleet soda lime was suspected when an ammonia-like odor was reported during its use in August 1992 (3). At that time, there was also concern that there might be amines present in the soda lime and questions arose regarding the relationship of contaminants to indicator dye. Preliminary screening of suspected lots of Sodasorb[®] by the Naval Medical Research Institute (NMRI) also revealed the presence of significant volatile hydrocarbons. Despite initial requests to the manufacturer for assistance and guidance, the issue remained unresolved. Therefore, in September 1992 the Chief of Naval Operations commissioned the Naval Medical Research and Development Command (NMRDC) to

investigate the issue. In October 1992 the responsibility for conducting the investigation was assigned to NMRI by NMRDC. The suspected contamination had a major impact on the U.S. Navy diving program when the Naval Sea Systems Command (NAVSEA) temporarily banned use of Sodasorb®. Sofnolime®, another brand of soda lime containing ethyl violet (Molecular Products Ltd., Essex, U.K.), was authorized as an interim replacement (4).

Our investigation was performed from Oct 1992 to May 1994 and had three main objectives:

- 1) Identify all potentially dangerous contaminants in Fleet soda lime.
- 2) Determine whether the indicator dye or the buckets are sources of contamination.
- 3) Recommend solutions to insure chemically safe soda lime for Fleet diving.

Preliminary findings, along with interim recommendations, have been reported previously to NAVSEA (5-7). A summary of our results and discussion of their significance for U.S. Navy diving are under review (8). In response, the U.S. Navy is expected soon to phase in, for all diving, non-indicating soda lime that will be required to meet specific contaminant limits (9). In contrast, this report provides expanded information on the analytical procedures used during the investigation and detailed results from all samples tested. Some of the information reported elsewhere (8) has been included here to produce a stand-alone document.

METHODS

Soda lime samples

Soda lime test samples were taken from 5-gallon buckets made of high-density polyethylene that were obtained from U.S. Navy supply stock or from the manufacturer:

1) High performance Sodasorb®

- a) 25 lots of 4-8 mesh granules
- b) with and without indicator dye
- c) packaged in 42 round buckets.

2) Sofnolime®

- a) 25 lots of 4-8 mesh granules (L grade) in 34 round or square buckets
- b) 3 lots of 8-12 mesh granules (D grade) in 5 square buckets
- c) all but 2 buckets containing indicator dye.

All Sodasorb® buckets were acquired full and unopened prior to testing; a few of the Sofnolime® buckets had been previously opened and partially used for Fleet diving. Buckets and lids were marked as to the month of their manufacture.

Sodasorb®

Sodasorb® samples were produced between April 1991 and July 1993, which included a number of lots without indicator dye made specifically for the Navy. A "lot" is all the soda lime made during one shift (\leq one day) of the manufacturing plant. Practically, there may have been more than one lot produced during a single day. Some of the non-indicator lots were packaged in canister bags that were then put inside the high-density polyethylene buckets (10 bags per bucket). Canister bags are laminated and comprised of paper, aluminum, and plastic. Each bag contains approximately 1350 g of soda lime. This bags-in-bucket packing was proposed by the manufacturer of Sodasorb® as an alternative packaging method.

All buckets and lids were made by one supplier (Bennett Industries, Inc., San Fernando, CA).

In addition to full buckets, a number of unused, empty buckets identical to those used for packaging soda lime were obtained and tested after lids had been in place for at least 1 week. **Appendix A** lists each Sodasorb® bucket that was sampled and discussed in this report by the following information: NMRI in-house bucket code, manufacturer lot number, date of soda lime manufacture (derived from lot number), and month of manufacture of bucket and lid (marked on the outside bottom of the bucket and inside lid surface). Lid dating required the removal of the lid from the bucket for a brief time (e.g., less than 30 s).

Sofnolime®

Sofnolime® samples were produced from September 1991 to December 1993. These included 2 buckets without indicating dye that were also made especially for the Navy. Sofnolime® was obtained either in round buckets (Fein Plastic Can Corp., currently a subsidiary of Bennett Industries) similar to those used for Sodasorb®, or in square containers (Blowmocan, Milton Keynes, U.K.), which had a removable cap on the pour spout rather than a lid. The round buckets were filled in the U.S. with absorbent, whereas the square buckets were packaged in the U.K. **Appendix B** lists each bucket of Sofnolime® in the same fashion as for Sodasorb®, except that lids were not removed. Therefore, lid dates are omitted.

Soda lime contaminant testing

Two types of contaminant tests were performed during this investigation: headspace testing and contaminant washout.

Headspace testing

Gas was initially sampled from the headspaces (gas spaces) inside closed buckets of

soda lime. These samples were subsequently analyzed to identify and quantify volatile organic compounds using gas chromatography (GC). Samples were taken using either 1) 500-ml stainless steel cylinders that had been previously heated and evacuated to at least 30 millitorr or 2) 150-ml passivated stainless steel syringes (Scientific Instrumentation Specialists, Moscow, ID). A specially designed stainless steel adaptor with an 18-gauge needle was inserted through the lid and penetrated approximately 3.5 cm into the bucket space. A rubber gasket at the base on the needle formed a tight seal preventing outside air from being drawn into the bucket during sampling. Preliminary GC testing showed that there was no observable effect of the adaptor on either ppm level hydrocarbon mixtures or hydrocarbon-free gas that were flowed through it. Headspace samples were drawn following attachment to the adaptor of either an evacuated cylinder or a syringe that had been purged 3 times with hydrocarbon-free gas. For cylinder sampling, the cylinder valve was opened slowly, 1 min then allowed for equilibration, and the valve closed before removal from the bucket. For syringe sampling, the syringe was filled with approximately 50 ml of bucket gas, expelled to the atmosphere, and then filled completely (150 ml) before removal.

Upon removal of the bucket adaptor, air was occasionally heard entering the bucket, suggesting that negative pressure had developed during sampling and that cylinders may have been equilibrated at less than atmospheric pressure. In other cases, leaks may have allowed laboratory air to enter the bucket during both cylinder and syringe sampling. For these reasons, levels of organic compounds measured in the headspace samples may be somewhat lower than actual concentrations inside the bucket prior to sampling. Following sampling, the needle hole in the bucket lid was sealed externally using a GC rubber septum and high-

strength adhesive-backed tape.

Headspace samples were not analyzed for ammonia and amines, because our method of analysis, using Fourier Transform Infrared (FTIR) spectroscopy, required a constant flow to produce reliable data as discussed below. No reliable method was developed to allow GC analysis of ammonia and amines.

Contaminant washout

A method was subsequently developed to measure contaminant washout as humidified 5% CO₂ in hydrocarbon-free air flowed through a bed of soda lime using the test apparatus shown in **Figure 1**. This apparatus, with a total internal chamber volume of 11.2 l, was designed to simulate conditions that might occur during actual use of the absorbent in order to help estimate exposures during diving. The gas was directed through the bed at 5 l/min, out through the gas cell where FTIR analysis occurred, and then vented into the atmosphere. Ammonia, amines, and total hydrocarbons were measured using FTIR; gas was also sampled for subsequent detailed analysis of volatile organic compounds by GC.

The test apparatus was loaded by first opening the bucket spout and pouring soda lime into a tared metal pan that was then covered with a metal lid and weighed to 2500 ± 10 g on a triple-beam or electronic balance. The bucket spout was immediately closed following soda lime transfer. Following weighing, the test apparatus was filled with the soda lime and sealed. These steps were completed quickly (generally less than 10 min) to minimize offgassing of the soda lime.

This was an open system where contaminants were flushed from the soda lime rather than allowed to recirculate as they might in a closed-circuit breathing device. As gas

continuously flowed through the FTIR, frequency of measurements was only limited by the time required for the FTIR quantitation program to complete 1 cycle, which was slightly less than 3 min. Soda lime samples were required to be analyzed in the test apparatus for at least 20 min; usually this time was considerably longer (e.g., over 1 h), until ammonia and amine levels dropped to below 1 ppm. Prior to gas sampling for GC analysis, an open-ended adaptor was attached to the normally closed sample point (noted in **Figure 1**). This allowed approximately 100 ml/min of gas leaving the soda lime bed to flush the sample line for 40 s. The cylinder or pre-flushed syringe was then attached and sampling done the same as for headspaces, except for one difference: the syringe was first purged with a full 150 ml of sample gas before obtaining the actual sample vs. only a 50-ml purge for headspace samples to minimize the amount of gas removed from the closed bucket.

During each experiment, flow, dew point, and CO₂ level were determined for both input and output gas, and ambient laboratory and soda lime bed temperatures measured. Dew point was measured using a dew point hygrometer (model Hygro-M1, General Eastern Instruments, Woburn, MA). Carbon dioxide was measured in the output gas with an infrared CO₂ analyzer (model LI-6252 with model LI-670 flow control unit, LI-COR, Inc., Lincoln, NE), calibrated with a primary standard of 500 ppm CO₂ in air, to insure that the absorbent had scrubbed all CO₂ from the gas. Ambient laboratory and soda lime bed temperatures were measured using a Tele-thermometer (model 2100; Yellow Springs Instrument Co., Yellow Springs, OH). Flows were monitored with rotameters found accurate to within 10% of reading by measuring the volume of water the gas flow displaced from a graduated cylinder over a known time.

Gas chromatography

Gas samples were screened for a broad range of volatile organic compounds using Shimadzu GC-9A temperature-programmable gas chromatographs (Shimadzu Corp., Columbia, MD) with both flame ionization detection (FID) and mass spectrometry (MS). For GC/FID, gas samples (0.5 ml) were introduced using gas sample valves. For GC/MS (model 5970 Mass Selective Detector, Hewlett-Packard Co., Rockville, MD), 100 ml of gas was preconcentrated at 10 °C on a solid multibed carbon adsorbent (carbotrap 300; Supelco, Inc., Bellefonte, PA). Subsequent thermal desorption at 340 °C for 5 min (Nutech 8533 Universal Sample Concentrator, Nutech Corp., Durham, NC) introduced the sample into the GC. Mass spectrometer scanning was from 20 to 200 amu.

The following columns were used with the indicated detectors and temperature profiles:

- 1) Vocol wide-bore capillary column, 30 m x 0.53 mm, 3.0 µm film. FID: 50 °C for 3 min, raised at 8 °C/min to 150 °C for 4.5 min. GC/MS: -20 °C for 3.1 min, raised at 20 °C/min to 150 °C for 11.4 min
- 2) Supelcowax 10 wide-bore capillary column, 60 m x 0.53 mm, 1.0 µm film. FID: 50 °C for 3 min, raised at 8 °C/min to 150 °C for 5 min.
- 3) 1/8 inch x 10 ft stainless steel packed column with 3% SP-1500 on 80/120 carbopack packing. FID: 40 °C for 1 min, raised at 20 °C/min to 200 °C for 4 min.

All columns were obtained from Supelco, Inc.

Prior to analysis, all samples in stainless steel cylinders, which were near atmosphere pressure, were vented to atmosphere and then pressurized with 15 psig of hydrocarbon-free air

using a low-delivery pressure, high-purity regulator. Samples could then be delivered to the instruments via positive pressure from the cylinder. Quantitation was adjusted for this dilution effect by applying a correction factor based on the pressures or by performing identical venting/pressurizing procedures with the calibration mixtures. Preliminary testing with calibration mixtures demonstrated that such procedures introduced an additional error of no more than several percent relative of expected value.

During the initial part of this investigation, the same gas sample was simultaneously analyzed by GC/FID and GC/MS on the same GC using identical columns (Vocol) in order to determine whether all organic species, particularly the more volatile ones, were effectively trapped and released by the carbotrap 300 adsorbent. Both analytical methods appeared to produce similar results in terms of numbers of peaks detected, the general appearance of the chromatograms, and quantitation, except as described below. Exact retention times were different, however, as would be expected since injection methods were different (i.e., gas valve injection vs. thermal desorption). Once this agreement was confirmed, GC/MS was used exclusively because of its increased sensitivity due to preconcentration and the ability to generate mass spectra to facilitate identification.

Identification of organic species was based on comparison of retention times of sample peaks and of species in commercially acquired, primary gas standards. All identifications were confirmed by comparison of mass spectra. Unknown compounds that did not match retention times of the standards were identified after careful review of library search results using Hewlett-Packard G1034B or G1034C software for the MS ChemStation (DOS series) with the NIST/EPA/MSDC 54K Mass Spectral Database and in view of the limitations inherent in

such searches. Unfortunately, for many of the components of the complex mixtures found in samples, it was not practical to confirm the search results by injection of the pure chemicals into the GC/MS.

Quantitation of volatile organic compounds by GC was based on GC/MS analysis with a conservative reporting limit of 0.1 ppm. When present, hexane, octane, and decane were individually quantified using a gravimetric standard, certified to \pm 2% relative, containing 2 ppm each of these compounds in hydrocarbon-free air (Scott Specialty Gases, Plumsteadville, PA). The remaining straight-chained and branched hydrocarbons that were found were expressed both in terms of octane and decane equivalents by quantifying the sum of their peak areas relative to the areas of octane and decane in the calibration standard. Concentrations expressed in these two ways were generally within 10-20% of each other. Other contaminants that were detected, but estimated to be below the reporting limit, were not quantified.

Thermal desorption from carbotrap 300, as used in this investigation during GC/MS, was not effective in removing all the heavy molecular hydrocarbons (decane and above). For this reason, GC/MS quantitation of the few samples with hydrocarbons levels much higher than the calibration standard (2 ppm) may be low by up to an estimated 30% relative. This estimate is based on 1) comparative GC/FID data, 2) comparative GC/MS data from direct loop injection, and 3) repeat thermal desorption and subsequent GC/MS analysis of a single sample to estimate the amount of residual hydrocarbons not desorbed during the first analysis. For quantitation of samples with hydrocarbon concentrations of several ppm or less, the error in quantitation is estimated to be less than \pm 20% relative.

Precision for GC/MS was checked at the start of each day by performing two consecutive analyses of 100 ml of a primary standard of 2 ppm each of Freon 113, methyl chloroform, benzene, toluene, and xylenes. Percent coefficients of variation (%CV) for peak areas, where $\%CV = 100 * (\text{difference between the 2 injections}) / (\text{mean of the 2 injections})$, were generally better than 10%. Gas Chromatography/MS responses were shown to be linear to within 10% over a range from 1 - 2 ppm each of hexane, octane, and decane. Detectability limits for GC/MS are estimated to be less than 0.05 ppm for hexane, octane, and decane.

FTIR analysis

FTIR analysis (model 1600 FT-IR, Perkin Elmer Corp., Norwalk, CT) with a 20-meter gas cell was used to measure ammonia, ethyl amine, diethyl amine, octane (as a measure of total hydrocarbons) and water, which was included to account for its interference in samples. The FTIR was calibrated each day using QUANT software supplied with the instrument, which applies curve-fitting to data from a defined spectra window rather than from one discrete frequency. Calibration used the following gravimetric standards obtained commercially from several sources and individually prepared in hydrocarbon-free air: 5 to 30 ppm ammonia, 5 and 10 ppm ethyl amine, 5 to 10 ppm diethyl amine, and 10 ppm octane, all certified to 2% relative, and 100% humidified air (hydrocarbon-free) for water. The actual ammonia standards used for calibration depended on the expected ammonia concentrations of the samples. When indicating dye was present in the soda lime, the higher ammonia standards (e.g., 10 ppm and 20 or 30 ppm) were used to quantitate the relatively high ammonia found. When indicating dye was absent, the lower ammonia standards (e.g., 5 and 10 ppm) were

used as little ammonia was usually found. In this case, only a single value for total hydrocarbons was derived from the infrared spectrum that was used to gauge washout time rather than to characterize the hydrocarbon profile that was done by GC. Amines were not measured during the early phase of this study, due to problems in method development.

All scans were ratioed to a background spectrum that was obtained at the start of each day after the gas cell had been evacuated to < 1 millitorr. Spectra were recorded with 2 cm⁻¹ resolution from 4000 to 700 cm⁻¹. Calibration windows for all components were set from 1200 to 800 cm⁻¹, except for octane, which was from 3050 to 2750 cm⁻¹. Each quantitation cycle was completed in less than 3 min and produced 1 measurement for each of the 5 components.

Performance testing and experience acquired through use of the FTIR with the 20-meter gas cell demonstrated the unreliability of measurements under no-flow conditions. Declines in analyte levels when flow was stopped was a particular problem with reactive species such as ammonia and the amines that presumably adsorbed to the interior surfaces of the gas cell. For these reasons, all calibration and analyses of the FTIR were done with gas flowing through the cell except for special testing where noted.

FTIR measurements for all analytes, except water, were shown to be linear to within 0.5 ppm absolute or better over the concentration ranges of the standards by using a precision gas divider (STEC model SGD-710, Horiba Instruments, Inc., Ann Arbor, MI). The STEC device allowed blending of the calibration gas with a diluent gas in 10 equal steps from 0% to 100% of the original concentration and has been previously shown to be linear to within the manufacturer's specification of $\pm 0.5\%$ of full scale, using 10 to 100 ppm gas standards of

several hydrocarbon species (10). Here, dry air, 100% humidified air, and the 30-ppm ammonia standard were all used as diluent gases with the gas flowing from the divider directly into the gas cell. The wet air and ammonia dilutions demonstrated that high water and ammonia, present during soda lime analysis, did not interfere with quantitation of the other analytes.

Recovery of octane, ammonia, and the two amines following passage through the apparatus was determined by flowing the calibration standards (dry) at 5 ℓ/min directly into the inlet side of the empty test chamber and measuring their concentrations in the 20-meter gas cell after 10 min. Measured concentrations in the cell were within 2% relative of the concentrations of the standards. Baseline composition of the test apparatus was also determined by flowing hydrocarbon-free air through the water bubbler and empty test chamber; during this octane, ammonia, and the two amines were monitored for at least 15 min. None of the 4 analytes were detected (< 0.1 ppm) with FTIR and no volatile organics were detected (< 0.05 ppm) with GC/MS.

Preliminary testing demonstrated that FTIR measurements reached 99% of full response after 3 quantitation cycles or less than 9 min following switching the gas cell initially filled with ammonia-free gas to a 5 to 30 ppm ammonia standard delivered at 5 ℓ/min . Similar response times were observed for washout to reach levels < 0.1 ppm when the reverse switch from ammonia standard to ammonia-free gas was done. The FTIR output was also monitored as a trace chloroform mixture (< 1 ppm octane equivalent) that was introduced into the empty soda lime vessel of the test apparatus was flushed out through the gas cell with 5 ℓ/min of hydrocarbon-free gas. Again, after less than 9 min, octane equivalent levels

fell below 0.1 ppm (FTIR). Complete removal of chloroform by this time was confirmed by analyzing gas samples with GC/MS.

The reliability of FTIR procedures for analysis of gas mixtures such as those from soda lime was also investigated by preparing "synthetic" gas mixtures inside the gas cell using the primary gas standards. Approximately equal partial pressures of 10 ppm octane, 10 ppm ammonia, 5 ppm of both ethyl and diethyl amines, and dry or 100% humidified air were added to the previously evacuated gas cell using a high-precision pressure gauge (models #122AA-00010AB and #122AA-01000AB, MKS Instruments, Inc., Andover, MA) to a final pressure of approximately 1.3 atm. All gas additions were completed within 5 min, and FTIR analysis was performed during the following 40 min under no-flow conditions. Measured values were compared to calculated values based on the partial pressure of each component added to the mixture. Measured values immediately after mixing were found to equal on average 99%/100% (using dry air/using wet air) of the calculated octane level, 91%/87% of ammonia, 111%/100% of diethyl amine, and 135%/113% of ethyl amine. These are based on 3 tests each with dry and wet air. Concentrations, especially those of ammonia, then dropped with time as analytes presumably interacted with the interior of the gas cell. By 40 min, measured octane (dry air/wet air) was 97%/99% of calculated value, ammonia 72%/71%, diethyl amine 93/91%, and ethyl amine 121%/92%.

Based on the preceding tests and experience using these procedures, FTIR lower reporting limits are conservatively defined as 0.5 ppm for octane, ammonia, and diethyl amine, and 1 ppm for ethyl amine. Analytical accuracy is conservatively estimated as \pm 0.5 ppm absolute for octane, ammonia, and diethyl amine, and \pm 1 ppm for ethyl amine for the

concentration ranges measured.

Dye testing

Soda lime samples were analyzed for ethyl violet dye, using methods we previously described (9), to help clarify the potential role that the dye played in the contamination problem. All dye measurements were performed in March-May 1994 following completion of testing for gaseous contaminants.

RESULTS

Test apparatus conditions

During washout experiments with the test apparatus in which 5 l/min of 5% CO₂ in air flowed through a 2500-gram bed of soda lime, the following conditions were recorded.

1) Laboratory temperature: 19-27 °C

2) Soda lime bed temperature:

Starting: laboratory temperature ± 0.5 °C

Post 60-min flow: 31-36 °C

3) CO₂ in output flow from soda lime bed:

< 0.01% for the duration of the test

4) Dew point

Input gas to soda lime bed: 0.5 °C below laboratory temperature

Output gas from soda lime bed: 19-21 °C

Volatile Organic Compounds

Sodasorb®

At least 3 different hydrocarbon profiles were found in headspace samples from Sodasorb® (**Table 1** and **Figure 2**). These profiles are defined based on the predominant species present:

- 1) Approximately 1 - 2 ppm of a range of hydrocarbons including hexane, octane, and decane, and < 1 ppm of a highly volatile species identified as a butene isomer by its mass spectra. The majority of Sodasorb® samples had this profile.
- 2) Approximately 30 - 60 ppm of a complex mixture of aliphatic hydrocarbons with 7 - 10 carbon atoms/molecule. This profile characterizes one lot of indicating Sodasorb® made in 1991.
- 3) Approximately 5-10 ppm of a complex mixture of aliphatic hydrocarbons with 9 - 13 carbon atoms/molecule and again < 1 ppm of the butene isomer. This profile characterized a) another lot of indicating Sodasorb® made in 1992, b) all the non-indicating Sodasorb® made in 1992 and 1993 that was tested, and c) "virgin" buckets made in 1 month in 1992, but never exposed to soda lime (NMRI's codes: GPE1 and GPE2). Buckets made the same month as these latter ones had been used for packaging some of the non-indicating Sodasorb®.

Headspace measurements repeated up to 6 months following the first samples generally agreed to within the level of analytical precision although the large difference in replicates for bucket N6 suggests an error in sampling. Hydrocarbon levels measured in gas flowing out of a bed of Sodasorb® were generally lower than those measured from the headspace of the same bucket (**Table 2**). Hydrocarbons more volatile than decane appear

easily removed by gas flow as they were absent or much reduced in washout samples.

The similarity in the hydrocarbon profiles of "virgin" buckets and Sodasorb® packaged in buckets made the same and different months suggests the bucket was the contaminant source. Unfortunately, older "virgin" buckets were not available to allow comparison with earlier lots of Fleet soda lime. However, our testing revealed another case where hydrocarbon profiles of the same lot of soda lime varied when packaged in buckets manufactured in different months (compare buckets BB4 and BB4D with buckets BB4C and BB4F in Table 2). The role of the bucket lids was not evident from the data, but would be confounded by the buckets.

In addition to the main contaminants distinguishing the profiles, most samples also contained low levels (< 0.1 ppm) of a number of other organic contaminants that were similar among all buckets. These included several alcohols and tetrahydrofuran. The majority of hydrocarbons may originate either from residual monomers from the bucket polymer or as processing aids used during the blow molding manufacturing process. These would include hexane, octane, decane, and the complex hydrocarbons mixtures. The alcohols (methanol, ethanol, and isopropyl) may be residual materials present in the Sodasorb® itself. Tetrahydrofuran and some of the other compounds may have been components of the ink used to mark the outside of the buckets.

Sodasorb® alternative packaging

Early test data by NMRI implicating the bucket as a problem prompted the manufacturer of Sodasorb® to propose an alternative packaging method using the canister bags described earlier. This was to be an interim packaging method (thus, all such buckets are

coded "IP#") until a long-term solution could be found. The 3-layer bags were to provide a barrier preventing or reducing hydrocarbon pickup by the soda lime from the bucket. Testing revealed that Sodasorb®, packaged this way and initially found to be hydrocarbon-free, slowly picked up hydrocarbon contaminants (profile 3) when the bags were placed into buckets. This resulted in absorbent gas concentrations of over 50% of bucket headspace concentrations after less than 3 months. This method was obviously not very effective in insuring hydrocarbon-free material over normal storage periods (e.g., several years).

Sofnolime®

At least 3 different hydrocarbon profiles were also found in headspace samples from Sofnolime® (**Table 3**):

- 1) Approximately 1 - 2 ppm of a range of hydrocarbons including hexane, octane, and decane, and < 2 ppm butene isomer. All Sofnolime® from round buckets had this profile, which appeared identical to Profile 1 of Sodasorb®.
- 2) Approximately 1 - 3 ppm of a mixture of aliphatic hydrocarbons with up to 10 carbon atoms/molecule. No hexane, octane, or decane were detected. Mass spectra search results suggested that many of the contaminants were unsaturated or cyclic in nature. Sofnolime® from all but one of the square containers had this profile.
- 3) Approximately 5 ppm of a complex mixture of aliphatic hydrocarbons with 7 - 9 carbons/molecule and < 0.5 ppm methyl chloroform. This profile characterizes one lot of Sofnolime® made in 1992 and packaged in square containers.

As with Sodasorb®, most samples also contained low levels (< 0.1 ppm) of a number of other organic contaminants, and hydrocarbon levels measured during washout were usually

lower than headspace concentrations.

Washout curves

Sodasorb®

Peak ammonia levels during washout from a test bed of indicating Sodasorb® ranged from 5 - 29 ppm, with an overall average of 17 ppm for samples taken from 23 buckets representing 16 different lots (**Table 4**). It generally took over 1 h of gas flow for ammonia to wash out to below 1 ppm. In a few instances, ammonia levels remained above 25 ppm for over 15 min. All detectable hydrocarbons (i.e., octane) were usually removed after 20 to 50 min of gas flow. Peak diethyl amine levels varied up to 4 ppm, with the washout curve generally tracking that of ammonia. Maximum ethyl amine levels ranged up to 2 ppm, although these values should be viewed in the context of an estimated analytical error of \pm 1 ppm, which is twice that of ammonia and diethyl amine.

Washout curves consistently show longer washout times for the polar ammonia and amines than for the hydrocarbons, which may be due the increased adsorbance of soda lime for these compounds. Retesting up to 3 days after washout produced no evidence of contaminant regeneration. Two samples from the same bucket run back-to-back on the same day produced peak ammonia values that were always within 5% of each other (**Table 5**) and washout curves that generally agreed within the level of analytical precision. Samples from the same bucket over a period of less than a year generally agreed to within several ppm ammonia and 1 ppm octane (**Table 4**). The few large declines in subsequent ammonia measurements can be attributed to loss of volatiles from buckets that were not effectively

sealed, as substantial drops in octane measurements also were noted. However, considerable variation in contaminant levels was often observed among buckets containing soda lime from the same lot. FTIR estimates of total hydrocarbons in octane equivalents agree well with the GC data despite the analytical error and differences in technique (compare GC data in **Table 2** with FTIR octane data in **Tables 4 and 6**).

No more than 1 ppm ammonia was detected in the gas during testing of non-indicator absorbent from 12 buckets representing 8 lots (**Table 6**). Peak diethyl and ethyl amine levels during washout from non-indicating Sodasorb® were both less than the respective reporting limits (0.5 and 1.0 ppm).

Sofnolime®

Peak ammonia levels during washout from indicating Sofnolime® ranged up to 3 ppm with an overall average of 1 ppm for samples taken from 25 buckets (**Table 7**). These ammonia levels were much lower than those found in indicating Sodasorb®. Washout curves for samples with several ppm ammonia were similar to those for Sodasorb®, although such washouts are poorly defined when ammonia levels are this low. Peak diethyl and ethyl amine levels in indicating Sofnolime® varied up to 3 and 1 ppm, respectively; these concentrations are similar to those from indicating Sodasorb®. No ammonia or amines above the reporting limits were measured in samples from the 2 buckets of non-indicator material.

Ethyl violet dye

Ethyl violet measurements for indicating soda lime ranged from 0.011 to 0.030% with no significant difference ($p < 0.01$) between Sodasorb® and Sofnolime®(**Tables 8-9**).

Unfortunately, dye analysis was done at the end of this investigation and only a few of the buckets containing indicating Sodasorb® were still left for testing. All buckets of soda lime supplied as non-indicator material were found to be free of dye as defined in reference (9).

DISCUSSION

Significant amounts of ammonia, ethyl and diethyl amines, and various aliphatic hydrocarbons were measured in gas samples from Fleet soda lime. The source(s) of the ammonia and amines are unknown, although one hypothesis is that they result from the breakdown of the indicator dye during manufacture, processing, or storage. This hypothesis is supported by the relatively low levels of ammonia and amines associated with all non-indicator soda lime as well by tests by one manufacturer of soda lime showing a strong correlation between dye decomposition and ammonia generation (1). Modeling results of these data suggest that peak diethyl amine concentrations and the amount of indicating dye in soda lime can be significant predictors of peak ammonia concentrations (8). However, it is interesting that amine and ethyl violet levels were similar in Sodasorb® and Sofnolime®, although ammonia levels were greatly different. Thus, contaminant relationships appear different for the 2 brands of soda lime. Such findings do not establish cause and effect, and it must be emphasized that a causative link between the indicator dye and such contamination has not yet been demonstrated.

All of the test samples of soda lime gave off varying amounts of aliphatic hydrocarbons which, in the case of recently manufactured Sodasorb®, appear to arise from the high-density polyethylene buckets. A direct link between buckets and hydrocarbon

contamination of past lots could not be made due to unavailability of older buckets never exposed to soda lime. However, the difference between Sofnolime® packaged in round vs. square buckets implicates the container as the source of the hydrocarbon contamination. Such contaminants are believed to come from the material used for construction of the bucket as was underscored by the experience with the canister bag packaging for Sodasorb®.

Samples of soda lime and buckets manufactured earlier than mid-1991 were unavailable, making the history of the contamination problem unclear. It is unknown whether the ammonia and/or amine problem with soda lime was the result of a change in the manufacturing process or past contamination had simply gone undetected. The variation in ammonia levels within and among soda lime lots may reflect, at least in part, differences in breakdown of indicator dye. Hydrocarbon contamination of soda lime was unexpectedly discovered during initial testing in response to the ammonia-like odor. Unfortunately, we have no information on what has occurred in the plant(s) that manufacture the high density polyethylene buckets that are used for soda lime.

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**Table 1. Hydrocarbon contaminants in bucket headspaces measured with GC.
Sodasorb® with and without indicator dye.**

NMRI Bucket Code	Hydrocarbon Profile	Test Date (month/year)	Butene (ppm)	Hexane (ppm)	Octane (ppm)	Decane (ppm)	Other HC's in Octane Equiv. (ppm)	Other HC's in Decane Equiv. (ppm)
A	1	10/92	0.3	0.3	0.4	0.4	1.0	1.2
B	2	11/92					34.0	40.3
WT1	2	12/92					43.1	50.6
		05/93					51.3	55.5
WT2	2	12/92					53.6	63.3
BB1	1	11/92	0.3	0.1	0.1	0.2	0.8	1.0
BB2	1	10/92	0.3	0.4	1.0	0.7	2.2	2.6
		11/92	0.2	0.2	0.4	0.7	2.0	2.4
BB3	1	10/92	0.2	0.3	0.1	0.1	0.8	1.0
BB4	3	11/92					9.9	11.7
		12/92					8.3	9.7
BB5	1	11/92	0.2	0.3	0.4	0.4	1.2	1.5
BB6	1	11/92		0.1	0.3	0.4	0.4	0.5
		11/92	0.1	0.2	0.3	0.3	0.3	0.3

NMRI Bucket Code	Hydrocarbon Profile	Test Date (month/year)	Butene (ppm)	Hexane (ppm)	Octane (ppm)	Decane (ppm)	Other HC's in Octane Equiv. (ppm)	Other HC's in Decane Equiv. (ppm)
BB7	1	11/92	0.3	0.2	0.2	0.3	0.8	0.9
		12/92	0.2	0.2	0.2	0.2	0.6	0.7
BB8	1	11/92	0.5	0.3	0.3	0.2	0.5	0.5
		12/92	0.4	0.4	0.2	0.3	1.6	1.9
BB9	1	11/92	0.2	0.2	0.1	0.2	1.3	1.5
		12/92	0.3	0.2	0.5	0.6	1.4	1.7
BB10	1	11/92	0.3	0.2	0.2	0.2	1.3	1.5
		12/92	0.2	0.2	0.5	0.6	1.4	1.7
G01	1	11/92	0.3	0.2	0.2	0.3	0.4	0.4
		12/92	0.2	0.5	0.2	0.2	0.5	0.6
G02	1	11/92	0.2	0.5	0.2	0.2	0.5	0.6
		12/92	0.2	0.6	0.3	0.2	0.6	0.7
G03	1	11/92	0.2	0.2	0.4	0.5	1.1	1.4
		11/92	0.1	0.3	0.3	0.3	0.5	0.6
G04	1	11/92	0.1	0.3	0.3	0.3	0.4	0.4
		12/92	0.2	0.2	0.2	0.4	0.7	0.9
G05	1	11/92	0.2	0.2	0.2	0.4	0.7	0.9
		12/92	0.2	0.2	0.4	0.5	1.1	1.4
G06	1	11/92	0.2	0.2	0.2	0.3	0.4	0.4
		12/92	1.3	0.3	0.4	0.4	0.9	1.1
G07	1	11/92	0.2	0.2	0.2	0.3	0.5	0.6
		12/92	0.1	0.3	0.3	0.3	0.4	0.4
G08	1	11/92	0.1	0.3	0.3	0.3	0.4	0.4
		12/92	0.5	0.3	0.1	0.1	0.4	0.4
G09	1	11/92	0.1	0.5	0.3	0.1	0.4	0.4
		12/92	0.2	0.6	0.4	0.2	0.6	0.7

NMRI Bucket Code	Hydrocarbon Profile	Test Date (month/year)	Butene (ppm)	Hexane (ppm)	Octane (ppm)	Decane (ppm)	Other HC's in Octane Equiv. (ppm)	Other HC's in Decane Equiv. (ppm)
G10	1	11/92		0.1	0.1	0.2	0.4	0.4
		12/92		0.1	0.2	0.2	0.2	0.3
N5*	3	01/93	0.1	0.2	0.1		3.0	2.7
		01/93	0.1	0.4	0.1		2.8	2.6
N6*	3	01/93	0.1	0.4	0.1		5.0	5.5
		05/93		0.1				
GPE1**	3	01/93		0.2	0.1		1.7	1.5
GPE2**	3	01/93		0.3	0.2		3.3	3.1
Range			<0.1-0.5	<0.1-1.3	<0.1-1.0	<0.1-0.7	0.2-53.6	0.3-63.3
N			38	38	38	38	38	38
#Buckets	28							

1. *: non-indicating soda lime.

2. **: empty bucket.

3. Profiles detailed in text.

4. Values are reported to the nearest 0.1 ppm.

5. Blank concentrations are <0.1 ppm.

6. Accuracy of analysis is estimated to be +/- 30% relative.

7. Other HC's: other hydrocarbons, in addition to the 4 individual species, expressed both in terms of octane calibration response and decane response. Only one value should be used to consider contaminant levels in gas.

Table 2. Hydrocarbon contaminants measured with GC after 5 min of flow during washout experiments. Sodasorb® with and without indicator dye.

NMRI Bucket Code	Source	Hydrocarbon Profile	Test Date (month/year)	Decane (ppm)	Other HC's in Octane Equiv. (ppm)	Other HC's in Decane Equiv. (ppm)
B	FS	2	05/93		12.4	13.5
WT2	FS	2	03/93		13.4	12.6
BB4	FS-1, B-1	3	04/93		3.6	4.6
BB4C	FS-1, B-2	1	05/93			
BB4D	FS-1, B-1	3	04/93		7.1	7.8
BB4F	FS-1, B-2	1	05/93			
BB6	FS	1	05/93	0.1		
BB8	FS	1	04/93	0.1		
G08	FS	1	05/93	0.2		
N7	NI	3	05/93		2.7	3.1
N13	NI	3	04/93		2.9	3.3
Range				<0.1-0.2	<0.1-13.4	<0.1-13.5
N				11	11	11

1. Butene, hexane, and octane levels <0.1 ppm for all samples.
2. Profiles detailed in text.
3. Source: FS = Fleet supply,
FS-1 = same lot of Fleet supply,
B-1 = bucket manufactured same month,
B-2 = bucket manufactured different month than B-1.
4. NI = non-indicating soda lime.
5. Values are reported to the nearest 0.1 ppm.
6. Blank concentrations are <0.1 ppm.
7. Accuracy of analysis is estimated to be +/- 30% relative.
8. Other HC's: other hydrocarbons, in addition to decane, expressed both in terms of octane calibration response and decane response. Only one value should be used to consider contaminant levels in gas.

**Table 3. Hydrocarbon contaminants in bucket headspaces measured with GC.
Sofnolime® with indicator dye.**

NMRI Bucket Code/ Bucket Type	Hydrocarbon Profile	Test Date (month/ year)	Butene (ppm)	Hexane (ppm)	Octane (ppm)	Decane (ppm)	Other HC's in Octane Equiv. (ppm)	Other HC's in Decane Equiv. (ppm)
SL2/R	1	12/92		0.5	0.4	0.4	0.5	0.6
SL3/R	1	12/92		0.5	0.5	0.4	0.4	0.4
SL4/S	2	12/92	0.3				3.5	3.8
SL5/S	2	12/92					1.3	1.4
SL6/S	2	12/92	0.5				3.1	3.4
SL7/S	2	12/92					0.9	0.9
SL8/S	2	12/92					0.7	0.8
SL9/S	2	12/92					1.2	1.3
SL15/R	1	03/94		0.1	0.5	0.3	1.1	0.8
SL16/R	1	03/94		0.2	0.5	0.5	1.2	1.7
SL18/R	1	03/94	0.9	0.9	0.9	0.4	2.2	2.0
SL20/S	2	03/94					0.2	0.2
SL23/S	2	03/94					0.7	1.0
SL25/S	2	05/94					0.3	0.2
SL27/S	3	03/94			1.2		4.8	4.4
SL32/R	1	03/94	1.6	0.4	0.5	0.3	1.1	1.6

NMRI Bucket Code	Hydrocarbon Profile	Test Date (month/year)	Butene (ppm)	Hexane (ppm)	Octane (ppm)	Decane (ppm)	Other HC's in Octane Equiv. (ppm)	Other HC's in Decane Equiv. (ppm)
SL33/R	1	03/94	0.9	0.7	0.4	0.2	0.9	1.3
SL34/R	1	05/94	1.0	0.4	1.0	0.3	1.8	1.6
SL35/R	1	03/94	1.3	0.5	0.7	0.3	1.1	1.6
SL36/R	1	04/94	0.4	0.5	0.3	0.2	0.9	0.9
SL37/R	1	04/94	0.6	0.4	0.3	0.2	0.8	0.8
Range			<0.1-1.6	<0.1-0.9	<0.1-1.2	<0.1-0.5	0.2-4.8	0.2-4.4
#Buckets	21							

1. Profiles detailed in text.
2. Values are reported to the nearest 0.1 ppm.
3. Blank concentrations are <0.1 ppm.
4. Accuracy of analysis is estimated to be +/- 30% relative.
5. Other HC's: other hydrocarbons, in addition to the 4 individual species, expressed both in terms of octane calibration response and decane response. Only one value should be used to consider contaminant levels in gas.

Table 4. Soda lime contaminants measured with FTIR during washout experiments.

Sodasorb® with indicator dye.

NMRI Bucket Code	Source	Test Date (month/year)	Ammonia (ppm)	Ammonia washout (min)	Diethyl amine (ppm)	Ethyl amine (ppm)	Octane (ppm)	Octane washout (min)
B	FS	04/93	19.4	>71			12.4	45
		05/93	18.6	100			12.1	51
		12/93	17.1	82	3.0	<1	6.6	21
		02/94	12.7	72	3.0	<1	5.4	18
BB1	FS-1	05/93	28.4	>87			1.1	36
		11/93	25.3	>92	3.0	<1	0.5	
BB2	FS-1	04/93	19.2	71			1.1	24
		11/93	18.0	76	1.5	<1	0.9	
		11/93	17.7	62	1.6	<1	2.2	5
BB3	FS-2	06/94	13.0	43	0.8	<1	<0.5	
BB4	FS-2	04/93	13.3	>56			7.2	>56
BB4C	FS-2	05/93	20.0	51			0.8	
		12/93	22.1	52	1.3	<1	<0.5	
BB4D	FS-2	04/93	14.4	34			6.2	>54
		11/93	16.4	33	1.8	<1	5.0	52
		11/93	14.3	42	1.3	<1	4.4	72

NMRI Bucket Code	Source	Test Date (month/year)	Ammonia (ppm)	Ammonia washout (min)	Diethyl amine (ppm)	Ethyl amine (ppm)	Octane (ppm)	Octane washout (min)
BB4F	FS-2	05/93	15.3	44				0.7
BB5	FS-3	07/94	13.8	63	1.0	1.4	<0.5	
BB6	FS-3	04/93	8.8	>55			0.5	
		11/93	7.5	62	1.1	<1	0.5	
		12/93	5.4	62	0.6	<1	<0.5	
BB8	FS	04/93	16.4	>59			<0.5	
		05/93	16.8	61			0.9	
		09/93	18.7	77	1.7	<1	<0.5	
		11/93	16.4	72	1.7	<1	<0.5	
BB9	FS-4	05/93	26.1	71			1.1	36
BB10	FS-4	05/93	24.8	71			1.7	46
		12/93	20.4	82	1.9	<1	0.8	
C1	FS	05/94	24.3	112	3.7	1.4	0.7	
		07/94	26.8	93	4.3	1.4	0.8	
G01	FS	05/93	10.7	61			0.6	
		12/93	10.6	62	0.9	<1	0.5	
G02	FS	05/93	18.3	71			0.6	
		12/93	19.8	72	1.3	<1	<0.5	

NMRI Bucket Code	Source	Test Date (month/year)	Ammonia (ppm)	Ammonia washout (min)	Diethyl amine (ppm)	Ethyl amine (ppm)	Octane (ppm)	Octane washout (min)
G03	FS	06/93	29.0	>76			1.5	38
		09/93	23.6	82	2.1	<1	0.9	
		01/94	25.1	72	2.2	1.8	1.0	1.3
G04	FS	04/93	14.1	56			0.6	
		05/93	14.1	62			0.8	
		12/93	15.7	>62	1.0	1.1	0.6	
G06	FS	05/93	17.7	70			0.9	
		05/93	15.7	76			0.6	
		01/94	18.4	72	1.4	<1	0.5	
G07	FS	06/93	12.5	>66			0.7	
		01/94	14.8	62	1.6	2.0	0.5	
		11/93	17.0	72	1.3	<1	0.7	
G08	FS	04/93	14.4	64			0.6	
		01/94	20.0	82	1.8	<1	<0.5	
		12/93	7.4	>63			0.9	
G09	FS	01/94	20.0	82	1.8	<1	<0.5	
G10	FS	06/93	13.4	52	0.9	<1	<0.5	

NMRI Bucket Code	Source	Test Date (month/year)	Ammonia (ppm)	Ammonia washout (min)	Diethyl amine (ppm)	Ethyl amine (ppm)	Octane (ppm)	Octane washout (min)
Mean (SD)			17.3 (5.4)		1.8 (0.9)			
Range			5.4-29.0	33-100	0.6-4.3	<1-2.0	<0.5-12.4	0-72
N			50	50	27	27	50	50
#Lots	16							
#Buckets	23							

1. Source: FS = Fleet supply,
 FS-1 = same lot of Fleet supply,
 FS-2 = same lot of Fleet supply,
 FS-3 = same lot of Fleet supply,
 FS-4 = same lot of Fleet supply.
1. Washout times = time to reach <1 ppm; > indicates washout not completed during experimental time.
2. Concentrations are maximum values during washout and are reported to the nearest 0.1 ppm.
3. Blank concentrations for amines indicate no measurement made.
4. Accuracy of analysis is estimated to be +/- 0.5 ppm for octane, ammonia, and diethyl amine, and +/- 1 ppm for ethyl amine.

Table 5. Intra-day precision for FTIR ammonia measurements of Sodasorb® with indicator dye.

NMRI Bucket Code	Test Date (month/year)	Maximum Ammonia Run 1 (ppm)	Maximum Ammonia Run (ppm)	Run1/Run2
B	12/93	17.1	16.8	1.02
BB1	11/93	25.3	26.0	0.97
BB2	11/93	18.0	17.6	1.02
BB4C	12/93	22.1	21.4	1.03
BB6	12/93	5.4	5.6	0.95
BB8	11/93	16.4	17.1	0.96
BB9	05/93	26.1	26.4	0.99
BB10	12/93	20.4	20.0	1.02
G01	05/93	10.7	10.7	1.00
"	12/93	10.6	10.9	0.98
G02	12/93	19.8	20.9	0.95
G03	01/94	25.1	24.6	1.02
G04	12/93	15.7	15.9	0.99
G06	01/94	18.4	18.9	0.97
G07	01/94	14.8	14.8	1.00
G08	11/93	17.0	16.9	1.01
G09	01/94	20.0	20.8	0.96
G10	12/93	7.4	7.4	1.01
Mean				0.99
Range				0.95-1.03
N				18

1. Runs 1 and 2 performed back-to-back on same day and completed in 3-6 h.

Table 6. Soda lime contaminants measured with FTIR during washout experiments.
sodasorb® without indicator dye.

NMRI Bucket Code	Test Date (month/year)	Ammonia (ppm)	Diethyl amine (ppm)	Ethy1 amine (ppm)	Octane (ppm)	Octane washout (min)
N7	05/93	0.6			3.3	16
N8	04/93	0.7			2.8	11
	05/93	0.8			3.1	16
N13	04/93	0.7			2.2	16
	12/93	0.9	<0.5	<1	2.5	13
IP2	07/93	0.6			1.0	
	09/93	<0.5	<0.5	<1	1.0	
IP3	07/93	1.1			0.7	
IP4	08/93	0.6			1.6	14
IP5	08/93	<0.5			1.0	
IP6	08/93	<0.5	<0.5	<1	<0.5	
IP8	09/93	<0.5	<0.5	<1	0.6	
IPIO	08/93	<0.5			0.9	
	12/93	<0.5	<0.5	<1	0.7	
IP12	08/93	<0.5	<0.5	<1	<0.5	

NMRI Bucket Code	Test Date (month/year)	Ammonia (ppm)	Diethyl amine (ppm)	Ethyl amine (ppm)	Octane (ppm)	Octane washout (min)
IP14	09/93	<0.5	<0.5	<1	1.2	17
Range		<0.5-1.1	<0.5	<1	<0.5-3.3	0-17
N		16	7	7	16	16
#Lots	8					
#Buckets	12					

1. Washout times = time to reach <1 ppm.
 2. Bucket code: IP2-IP14 contained soda lime in canister bags.
 3. Concentrations are maximum values during washout and are reported to the nearest 0.1 ppm.
 4. Blank concentrations for amines indicate no measurement made.
 5. Accuracy of analysis is estimated to be +/- 0.5 ppm for octane, ammonia, and diethyl amine, and +/- 1 ppm for ethyl amine.

Table 7. Soda lime contaminants measured with FTIR during washout experiments.
Sofnolime® with and without indicator dye.

NMRI Bucket Code	Test Date (month/year)	Ammonia (ppm)	Ammonia washout (min)	Diethyl amine (ppm)	Ethyl amine (ppm)	Octane (ppm)
SL3	10/93	0.7		<0.5	<1	0.6
SL3	10/93	0.7		<0.5	<1	0.5
SL3	02/94	0.7		<0.5	<1	0.6
SL12	02/94	1.7	52	1.1	1.1	0.7
SL13	02/94	1.1	18	0.9	1.0	0.7
SL14	02/94	2.0	42	<0.5	<1	0.5
SL15	02/94	1.1	17	<0.5	<1	0.8
SL16	02/94	1.0	19	<0.5	<1	0.7
SL17	02/94	0.8		<0.5	<1	0.7
SL18	02/94	0.5		0.9	1.2	0.6
SL19	02/94	1.3	42	<0.5	<1	<0.5
SL20	02/94	1.0		<0.5	<1	<0.5
SL21	02/94	2.7	72	3.4	1.0	<0.5
SL22	02/94	2.9	63	1.4	1.0	<0.5
SL23	02/94	0.5		<0.5	<1	<0.5

NMRI Bucket Code	Test Date (month/year)	Ammonia (ppm)	Ammonia washout (min)	Diethyl amine (ppm)	Ethyl amine (ppm)	Octane (ppm)
SL24	02/94	0.6		<0.5	<1	<0.5
SL25	02/94	1.9	52	0.5	<1	<0.5
SL26	02/94	1.7	52	0.6	<1	<0.5
SL27	02/94	1.0		<0.5	<1	0.6
SL28	02/94	1.0	21	<0.5	<1	<0.5
SL29*	02/94	<0.5		<0.5	<1	<0.5
SL30	02/94	0.7		<0.5	<1	<0.5
SL31*	03/94	<0.5		<0.5	<1	<0.5
SL32	03/94	2.0	53	0.6	<1	0.6
SL33	03/94	2.2	52	0.6	1.4	<0.5
SL34	03/94	2.5	63	2.1	1.3	0.6
SL35	03/94	2.2	52	0.6	1.3	0.6
Range	10/93-03/94	>0.5-2.9	0-72	<0.5-3.4	<1-1.4	<0.5-0.8
N		27	15	27	27	27
#Lots	19					
#Buckets	25					

1. Washout times = time to reach <1 ppm.
2. Concentrations are maximum values during washout and are reported to the nearest 0.1 ppm.
3. Accuracy of analysis is estimated to be ± 0.5 ppm for octane, ammonia, and diethyl amine, and ± 1 ppm for ethyl amine.
4. *: reduced dye

Table 8. Ethyl violet dye concentrations of Sodasorb® with indicating dye.

NMRI Bucket Code	Test Date (month/year)	Ethyl violet (%)
BB1	05/94	0.018
BB3	05/94	0.015
BB4D	05/94	0.011
BB5	05/94	0.016
C1	05/94	0.030
G01	05/94	0.018
G02	05/94	0.021
G09	05/94	0.027
G10	05/94	0.018
Mean (SD)		0.019 (0.006)
Range		0.011-0.030
N		9
#Lots		9

Table 9. Ethyl violet dye concentrations of Sofnolime® with indicating dye.

NMRI Bucket Code	Test Date (month/year)	Ethyl violet (%)
SL3	03/94	0.018
SL10	04/94	0.017
SL11	04/94	0.020
SL12	04/94	0.022
SL13	04/94	0.019
SL14	04/94	0.022
SL16	03/94	0.018
SL17	04/94	0.016
SL20	04/94	0.023
SL22	04/94	0.022
SL23	04/94	0.019
SL26	04/94	0.022
SL27	04/94	0.021
SL28	04/94	0.023
SL30	04/94	0.024
SL32	04/94	0.023
SL33	04/94	0.022
SL34	04/94	0.026
SL35	03/94	0.022
SL36	04/94	0.022
SL37	04/94	0.023
SL38	04/94	0.023
SL39	04/94	0.022

NMRI Bucket Code	Test Date (month/year)	Ethyl violet (%)
Mean (SD)		0.021 (0.002)
Range	3/94-4/94	0.016-0.026
N		23
#Lots		23

Appendix A. Sodasorb® buckets tested.

NMRI Bucket Code	Manufacturer Lot Number	Lot Date (month/ year)	Bucket Date (month/ year)	Lid Date (month/ year)
A	AA04-4004-12	04/91	01/91	03/91
B	AA08-4004-29	08/91	07/91	08/91
WI1	"	08/91	07/91	07/91
WI2	"	08/91	07/91	07/91
BB1	AB02-4004-28A	02/92	01/92	01/92
BB2	"	02/92	12/91	01/92
BB3	AB02-4004-06	02/92	12/91	11/91
BB4	"	02/92	10/91	11/91
BB4C	"	02/92	12/91	11/91
BB4D	"	02/92	10/91	11/91
BB4F	"	02/92	12/91	11/91
BB5	AB05-4004-18	05/92	12/91	03/92
BB6	"	05/92	03/92	03/92
BB7	AB05-4004-28	05/92	12/91	03/92
BB8	"	05/92	12/91	03/92
BB9	AB02-4004-28	02/92	12/91	01/92
BB10	"	02/92	12/91	01/92
C1	AB04-4004-28A	04/92	12/91	
G01	AB07-4004-6A	07/92	03/92	03/92
G02	AB07-4004-9	07/92	07/92	03/92
G03	AB06-4004-5	06/92	12/91	03/92
G04	AB07-4004-7	07/92	03/92	03/92
G05	"	07/92	03/92	03/92
G06	AB06-4004-2	06/92	12/91	04/92
G07	AB06-4004-9	06/92	03/92	04/92
G08	AB07-4004-6	07/92	07/92	03/92

NMRI Bucket Code	Manufacturer Lot Number	Lot Date (month/ year)	Bucket Date (month/ year)	Lid Date (month/ year)
G09	AB07-4004-8A	07/92	07/92	07/92
G10	AB07-4004-8	07/92	03/92	03/92
N5*	AB11-B600-19	11/92	11/92	11/92
N6*	"	11/92	11/92	11/92
N7*	"	11/92	09/92	12/92
N8*	"	11/92	09/92	12/92
N13*	"	11/92	09/92	12/92
IP2*, cb	AC06-4036-30	06/93	06/93	
IP3*, cb	AC07-4036-1	07/93	06/93	
IP4*, cb	AC06-4036-30	06/93	06/93	06/93
IP5*, cb	AC07-4036-2	07/93	06/93	06/93
IP6*, cb	AC07-4036-28	07/93	07/93	07/93
IP8*, cb	AC07-4036-12	07/93	07/93	06/93
IP10*, cb	AC07-4036-9	07/93	07/93	
IP12*, cb	AC07-4036-15	07/93	07/93	07/93
IP14*, cb	AC07-4036-2	07/93	06/93	06/93
GPE1**			09/92	11/92
GPE2**			09/92	11/92
Range		04/91- 07/93	01/91- 07/93	03/91- 07/93
#Lots	25			
#Buckets	44			
1. *: non-indicating soda lime. 2. **: empty bucket. 3. cb: packaged in canister bags which were then put inside the bucket.				

Appendix B. Sofnolime® buckets tested.

NMRI Bucket Code/ Bucket Type	Manufacturer Lot Number	Lot Date (month/year)	Bucket Date (month/year)
SL1/S	165092	09/92	06/92
SL2/R	005102G	10/92	
SL3/R	"	10/92	10/92
SL4/S	260091	09/91	
SL5/S	165092	09/92	
SL6/S	479111	11/91	
SL7/S	956072	07/92	
SL8/S	166092	09/92	
SL9/S	"	09/92	
SL10/R	004102G	10/92	10/92
SL11/R	006102G	10/92	10/92
SL12/R	007102G	10/92	10/92
SL13/R	018112G	11/92	10/92
SL14/R	198063G	06/93	05/93
SL15/R	016112G	11/92	10/92
SL16/R	"	11/92	10/92
SL17/R	134043G	04/93	12/93
SL18/R	"	04/93	12/93
SL19/S	166092	09/92	06/92
SL20/S	"	09/92	06/92
SL21/S	165092	09/92	06/92
SL22/S	"	09/92	06/92
SL23/S**	607033	03/93	12/92
SL24/S**	"	03/93	12/92
SL25/S**	348112	11/92	03/92

NMRI Bucket Code/ Bucket Type	Manufacturer Lot Number	Lot Date (month/ year)	Bucket Date (month/ year)
SL26/S**	348112	11/92	03/92
SL27/S	120082	08/92	03/92
SL28/S	119082	08/92	03/92
SL29*/S	522013	01/93	06/92
SL30/S	118082	08/92	06/92
SL31*/S**	323123	12/93	12/92
SL32/R	166053G	05/93	11/92
SL33/R	170053G	05/93	04/93
SL34/R	163053G	05/93	11/92
SL35/R	162053G	05/93	11/92
SL36/R	172053G	05/93	04/93
SL37/R	169053G	05/93	04/93
SL38/R	038122G	12/92	11/92
SL39/R	145043G	04/93	01/93
Range		09/91- 12/93	03/92- 12/93
#Lots	28		
#Buckets	39		

1. Bucket type: S = square, R = round.
 2. *: non-indicating soda lime.
 3. **: 8-12 mesh granules; all other
 buckets 4-8 mesh.

FIGURE LEGENDS

Figure 1. Diagram of test apparatus for measuring contaminant washout as humidified gas flowed through a bed of soda lime. The standard open-circuit test flushed contaminants from the system. The special closed-circuit test recirculated the gas flowing out of the soda lime back into the bed using a stainless steel bellows pump; this application is not discussed in this report.

Figure 2. Volatile hydrocarbon contaminants found in the headspace of Sodasorb® buckets. Representative ion chromatograms (GC/MS data; 35 to 200 amu) of the 3 different hydrocarbon profiles found in buckets containing Sodasorb® and profile 3 found in a "virgin" bucket that never was exposed to soda lime.

FIGURE 1

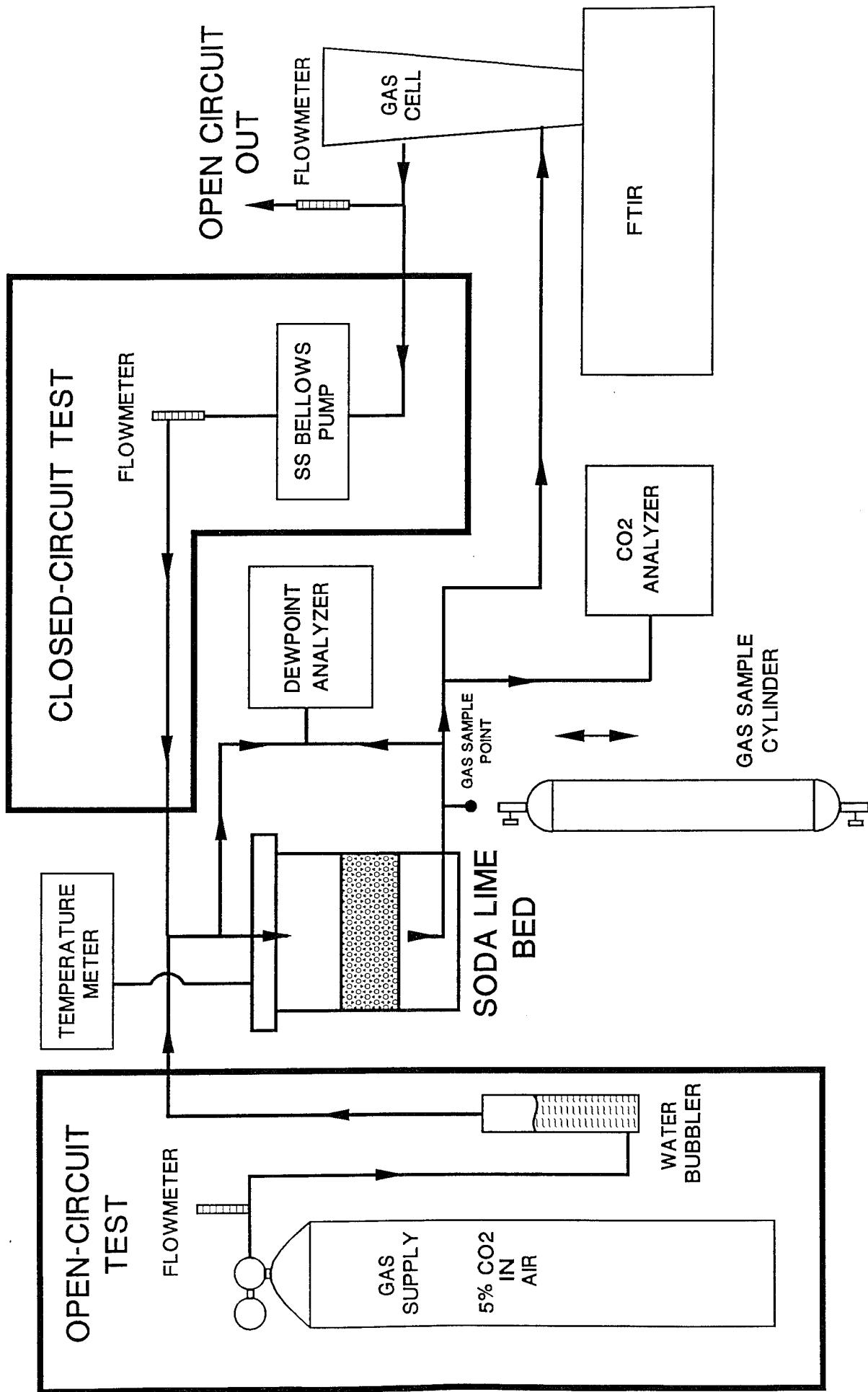
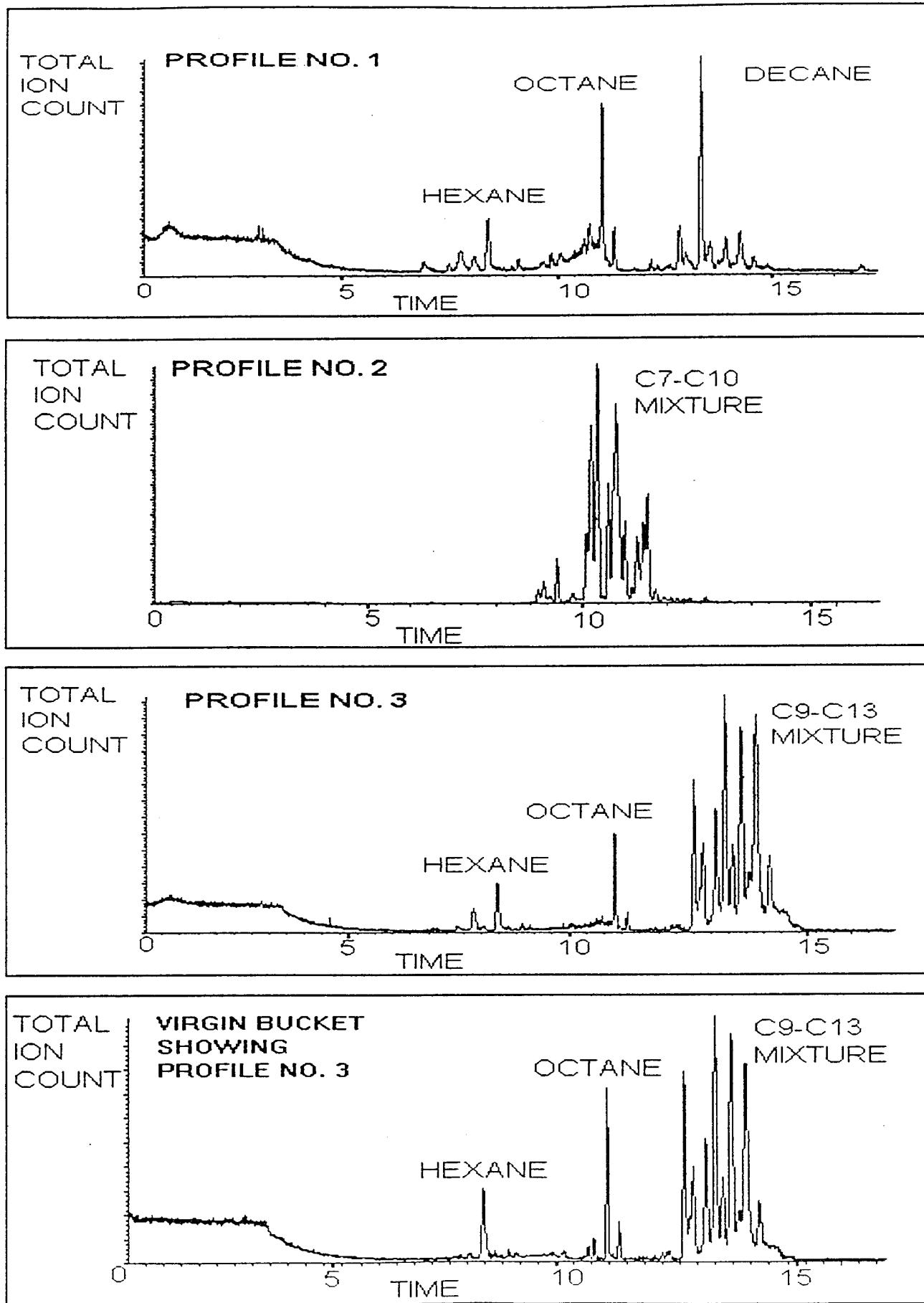


FIGURE 2



BETA AMYLOID PEPTIDE (1-42) DEPRESSES EVOKED ACETYLCHOLINE RELEASE IN CULTURED CILIARY GANGLION NEURONS. S. Miller and D. B. Gray*, Department of Biology, Simmons College, 300 The Fenway, Boston, MA 02115.

The avian ciliary ganglion is made up of cholinergic neurons and contains a subset of cells expressing somatostatin. This is also true of presynaptic neurons in the human CNS which lose cholinergic content and die as a result of Alzheimer's Disease (AD). Ciliary ganglion neurons also innervate the iris which has been recently used in an early diagnostic test for AD. Recent evidence has implicated the presence of mature neuritic plaques containing insoluble beta amyloid peptides as a precursor to neuronal degeneration associated with AD. In this study, the beta amyloid peptide fragment 1-42 was 'aged' for 7 days and then incubated for 20 hours at a concentration of 25 μ M with cultured stage 40 embryonic chick ciliary ganglion neurons. At this concentration aged 1-42 peptide solution clearly contained insoluble crystals which decorated the neurons. Peptides were added 30 min after neuronal plating to prevent effects on cell-substrate adhesion. Control wells either had no peptide or were incubated with amyloid fragment 1-28 which has not been reported to have any toxic effects. Acetylcholine (ACh) release was measured with labeled choline and evoked by 60 mM potassium-Tyroses. The presence of beta peptide 1-42 blocked 70-90 % of K-evoked release relative to either control. Interestingly, only 10 % of this dramatic effect could be accounted for by decreases in uptake of the precursor, tritium-labeled choline chloride. This *in vitro* preparation can be used to investigate cellular mechanisms underlying degradation of cholinergic function as well as neurotoxicity. Supported by The Simmons Fund for Research.

194.13

IN VIVO EFFECTS OF AMYLOID PEPTIDES LOCAL INJECTION ON RAT FOREBRAIN CHOLINERGIC NEURONS: MORPHOLOGICAL, NEUROCHEMICAL AND BEHAVIORAL STUDIES. L. Giovannelli, F. Casamenti, C. Scali, L. Bartolini and G. Peppe*, Dept. Pharmacol., Univ. Florence, ITALY 50134.

The extracellular deposition of β -amyloid ($\text{A}\beta$) in senile plaques is one of the neuropathological hallmarks of Alzheimer's disease (AD) and might be crucial to initiate the associated neuronal degeneration. As the cholinergic system is severely compromised in AD, we have investigated the effects of $\text{A}\beta$ on cholinergic forebrain neurons by injecting 10 μg of $\text{A}\beta$ fragments 1-40, 25-35 or scrambled 25-35 into the right nucleus basalis (NB) of 3 month-old Wistar rats. At different times, cortical ACh output was studied by microdialysis, cognitive function was assayed by passive avoidance and object recognition (OR) tests, and the brains were prepared for histology. Congo Red staining showed a birefringent deposit of aggregated peptide at the injection sites of β 1-40 and 25-35, but not of the scrambled, peptides. However, while the β 25-35 deposit lasted for about 2 wks, the β 1-40 one was detectable up to at least 4 mo. A decrease in the number of cholineacetyltransferase (ChAT)-immunoreactive neurons in the NB was also found 1 wk after injection of all peptides including the scrambled. However, after 2 mo such a decrease was only detectable in the β 1-40-injected rats (-30%), while a complete recovery of ChAT IR was observed following the other treatments. The reduction in ChAT IR was paralleled by a decrease in ACh output from the parietal cortex ipsilateral to the injection. While no major peptide effect was observed on passive avoidance up to 2 mo post-surgery, disruption of OR was brought about by β 25-35 in the first wks and by β 1-40 starting 2 mo post-surgery. The effects of β 1-40 on ChAT IR, ACh release, neurite morphology and cognitive function are currently being evaluated at longer times (4 to 6 mo). The results so far obtained indicate that the deposition of $\text{A}\beta$ may contribute to the cholinergic deficit associated with AD. Furthermore, rats injected with the β 1-40 peptide may be a useful model to study the long term effects of $\text{A}\beta$ deposition in the brain. Supported by a CNR grant: Target Project on Aging.

194.15

EFFECT OF β -AMYLOID PEPTIDE INJECTIONS ON MUSCARINIC RECEPTORS IN RAT HIPPOCAMPUS. Neelam Narang*, Neuropsychiatric Research Institute, 700-1st Ave. South, Fargo, ND, 58103; Departments of Neuroscience, Pharmacology & Toxicology, University of North Dakota, School of Medicine, Grand Forks, ND 58202.

Deposits of beta amyloid peptide (BAP), and degenerated neurons are some of the characteristics of patients with Alzheimer's disease (AD). This peptide has been shown to be neurotoxic when injected into rat hippocampus or incubated in cell cultures. However, the mechanism by which this neurodegeneration occurs is not known. In addition, many receptors, particularly muscarinic receptors are known to be altered in AD patients and aging. In order to examine whether the neurotoxicity induced by amyloid peptide affects the muscarinic receptors, we injected beta amyloid 1-40 fragments in rat hippocampus and examined changes in various subtypes of muscarinic receptors using autoradiography and *in situ* hybridization techniques. One group of Long Evans rats were unilaterally injected with 3 nmols (2 μl) of BAP fragments in the hippocampus, whereas the second group was injected with the vehicle in the same coordinates.

A significant decrease (11-23%) in [^3H]QNB binding in all hippocampal layers were detected in the lesioned rats, when compared to controls. Both M₁ receptor binding (labeled with [^3H]pirenzepine) and m₁mRNA were reduced (8-16%) in CA1, CA2, CA3, and dentate gyrus of the hippocampus of the lesioned brains. Similarly, a significant decrease in [^3H]AFDX-384 binding, but no change in m₂mRNA was observed in the lesioned rats. m₂mRNA was also reduced in dentate gyrus and CA1 region of the hippocampus. However, no difference was found in m₃mRNA in any of the hippocampal layers. The data suggest that β amyloid peptide are neurotoxic and may modulate muscarinic receptors and the mRNA encoding some of the receptor subtypes.

194.12

THE AMYLOID- β PROTEIN REDUCES THE INTRACELLULAR LEVELS OF ACETYLCHOLINE IN A MURINE SEPTAL CELL LINE, SN56. W.A. Pedersen and J.K. Blusztaaj*, Department of Pathology, Boston Univ. School of Medicine, Boston, MA 02118.

$\text{A}\beta$, a 39-43 amino acid fragment of the β -amyloid precursor protein, has been implicated in the pathogenesis of Alzheimer's disease (AD), but a relationship between reduced expression of cholinergic markers in AD and $\text{A}\beta$ deposition has not been established. Treatment of SN56 cells with $\text{A}\beta$ 1-28, 25-35, 1-40 and 1-42 reduced the intracellular levels of acetylcholine (ACh) as determined by HPLC. $\text{A}\beta$ 1-28 at a saturating concentration of 50 pM resulted in a 33% decrease in the levels of ACh after 48 hours, whereas $\text{A}\beta$ 1-42 caused a reduction of approximately 40% over the same period when administered at a saturating concentration of 100 nM. In an experiment where several peptides were tested at a concentration of 10 nM for 48 hours, $\text{A}\beta$ 1-40 was found to be only 50% as effective as $\text{A}\beta$ 1-42 at reducing the intracellular levels of ACh in SN56 cells, $\text{A}\beta$ 1-28 was as effective as $\text{A}\beta$ 25-35, and $\text{A}\beta$ 1-16 was inactive. We also observed that treatment of the cells with $\text{A}\beta$ 1-28 (50 pM) or $\text{A}\beta$ 1-42 (5 nM) for several weeks caused no further reduction in ACh levels. No cytotoxicity of the peptides was observed at the concentrations tested (up to 1 μM). Our results suggest that $\text{A}\beta$ suppresses the cholinergic phenotype of septal neurons at sub-toxic concentrations, an event which may precede the degeneration of these neurons in AD and may contribute to the loss of memory and learning functions characteristic of this disease.

(Supported by AG09525)

194.14

TIME COURSE ANALYSIS OF ACETYLCHOLINE NEUROTRANSMISSION AFTER INJECTION OF N-METHYL-D-ASPARTATE INTO THE NUCLEUS BASALIS MAGNOCELLULARIS. P.A. Shea*, T.M. Kerr, S.T. Ahlers, W. Wallace*, and V. Haroutunian, Naval Med. Res. Inst., Bethesda, MD; Natl. Inst. Aging, Baltimore MD, and Bronx VA, Bronx, NY.

Administration of N-Methyl-D-Aspartate (NMDA) into the Nucleus Basalis Magnoceularis (NBM) produces a reduction in ACh and subsequent increase in β -amyloid precursor protein (β -APP). We have previously demonstrated that brief hypofunction of the NBM after infusion of the local anesthetic lidocaine is sufficient to increase cortical β -APP. In order to further characterize the relationship of ACh to β -APP, we measured ACh before, during, and after infusion of NMDA in anesthetized rats in which 3mm microdialysis probes had been inserted on the contralateral and/or ipsilateral side. 2 μl of 50mM NMDA was infused into the NBM at a rate of 0.5 $\mu\text{l}/\text{min}$. Rats were maintained at a surgical plane of anesthesia with an initial dose of 400 mg/kg chloral hydrate (CH) and supplemental doses as needed for a period of 6-8 hours. For dialysis sampling beyond 8 hours, rats were lesioned, allowed to recover, re-anesthetized, and then sampled over several hours. The flow rate was 1.5 $\mu\text{l}/\text{min}$ and samples were collected every 20 minutes. Cerebral spinal fluid (CSF) was collected from the cisterna magna after rats were given a euthanizing dose of CH. Infusion of NMDA into the NBM resulted in a rapid 200% increase of ACh on the ipsilateral side. This increase was sustained for six hours and then decreased to below baseline levels thereafter. The increases and subsequent decreases in cortical ACh corresponded to specific alterations in β -APP measured in CSF. These data demonstrate the functional relationship between ACh and β -APP.

194.16

REDUCTION OF TYROSINE HYDROXYLASE AND GLUTAMATE IN THE LOCUS COeruleus AS WELL AS CHOLINE ACETYLTRANSFERASE IN THE MEDIAL SEPTUM FOLLOWING THE INTRAHIPPOCAMPAL INJECTION OF β -AMYLOID PEPTIDE (25-35). Charles D. Barnes* and Shiou-Yi Chen, Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, Pharmacology/Toxicology Program, Washington State University, Pullman, WA 99164-6520.

Alzheimer's disease is associated with the presence of β -amyloid plaque and cell loss in the cortex and certain subcortical structures. To investigate if the subcortical changes are due to the accumulation of β -amyloid protein (BAP), the major constituent of neuritic plaques, in the cortex, multiple injections of β AP fragment β (25-35) (1 or 3 nmol, 1 $\mu\text{l}/\text{site}$, 3 injection sites) were administered into rat left hippocampal area CA1-3 and dentate gyrus once a week for one or two weeks. The immunoreactivities of tyrosine hydroxylase (TH) and glutamate (Glu) in the locus coeruleus (LC) as well as choline acetyltransferase (ChAT) in the medial septum (MS) were studied at 1-week or 3-month survival times.

One week following the intrahippocampal injection, in addition to the hippocampal tissue damage, one injection of β (25-35) at either dose also substantially reduced the ChAT immunoreactivity in the MS and repetitive injections of 3 nmol of β (25-35) decreased the TH and Glu expressions in the LC. The loss of ChAT neurons was in a dose-dependent manner. Moreover, the severity of these effects was progressive over time. At 3-month survival time, the hippocampal lesions progressed to a remarkable loss of dorsal hippocampus and atrophy of cerebral cortex as well as enlargement of the ventricles. The enzyme expressions in the MS and LC were further reduced at this time point. These results suggest that accumulation of BAP in the cortex over time could lead to neuropathological changes in the subcortical regions.

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Toxicology Detachment
Dayton, OH**

Profile

Occupational and Environmental Toxicology in the Military Tri-Service Toxicology

David R. Mattie, Lana Martin and John M. Frazier

Military toxicology has many of the same problems and concerns found in the general field of toxicology. However, military toxicology is also faced with unique hazards associated with substances that are used primarily by the military services or that present an unusual type of exposure as a result of a military environment. The term 'environment' to a military toxicologist may mean the cockpit of an aircraft, the inside of a submarine (see cover photo) or battle tank, a restoration site on a base or post, a maintenance facility, a flightline or a flight deck or the environment of a battlefield during actual combat. Public concern and legislation have caused the military toxicologist to expand the traditional approaches for determining chemical hazards in all of these environments and to seek novel ways for applying toxicology to risk assessment. The military will continue to develop new materials and weapon systems to meet the needs of national security. Military toxicology will continue to define the toxic hazards associated with the fuels, chemicals and structural materials in advanced weapon systems. Methods are constantly being developed or refined to screen chemicals and materials as early as possible in the development process. This involves new *in vitro* techniques, tiered approaches, computer modelling and a thorough understanding of the basic mechanisms of action at all levels of interaction in the body.

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Historical perspective

Military toxicology evolved as a result of the World Wars. The United States began studying chemical warfare agents upon entering World War I out of concern for battlefield exposures. These studies were directed by the medical and pharmacology/toxicology sections of the US Army Chemical Warfare Service. On the home front, the war spawned tremendous growth in the US munitions industry. Casualties and fatalities from occupational diseases were attributed to exposure to nitrous gases in US munitions plants. Nearly 230 fatalities occurred per billion pounds of explosives manufactured as a result of occupational health hazards, underscoring the importance of industrial hygiene. In response, the US Army established the US Army Industrial Hygiene Laboratory at Johns Hopkins University, Baltimore, MD, USA. This laboratory played a major role in significantly reducing the number of occupational disease-related fatalities in World War II. In fact, the number of occupational disease-related fatalities in World War II was 46 times lower than in World War I.

World War II expanded toxicological issues beyond that of the munitions industry. The growth in the US chemical and automotive industries during the war years created many unique chemicals. Military personnel were continually coming into contact with new chemical compounds that were intended to operate and protect their machinery and, in some geographical locations, protect them from disease-carrying insects and pathogens. The continual development of chemical warfare agents increased the need for toxicological data to assess the physiology, pathology and therapy of chemical warfare injury.

By the 1950s, continued growth in the chemical industry, the introduction

of increasingly complex weapon systems, and the potential of mission degradation resulting from health hazards triggered the need for a Navy toxicology program. Fleet personnel were continuously exposed to chemical substances used aboard Navy submarines and ships including fuels, fuel additives, propellants, hydraulic fluids and lubricants. Many toxicological questions were raised concerning man/machine interface problems involved with nuclear submarine habitability. Thus, the Navy Toxicology Unit was established in 1958 at the National Naval Medical Center in Bethesda, MD. This unit initiated many military-unique toxicological study practices that were later taken over by the toxicology community at large and are still used today, such as continuous exposure studies and the 90-day study practice which was designed to correspond to the standard duration of submarine missions. In 1977 the Naval Medical Research Institute created the Toxicology Detachment at Wright-Patterson Air Force Base (AFB) to unite Navy personnel and resources with those of the Air Force. This relationship proved successful and established the foundation for Tri-Service Toxicology.

The Air Force toxicology program began in 1956. Research toxicologist Anthony A. Thomas, MD, studied the occupational toxicology of missile propellants and oxidizers. He and his staff of four were assigned space in the basement of the Wright-Patterson AFB Area B hospital dispensary. In 1959, the Aerospace Medical Laboratory became the focal point of the Air Force initiative 'Man in Space'. This initiative required limits for continuous human exposure in space travel. Dr Thomas moved the programme into Area B's Building 79, its current location, and designed an inhalation toxicology program to build a continuous exposure data base.

The increased awareness of adverse health effects from exposure to chemicals, coupled with increasingly complex military exposure situations, emphasised the need for a broader toxicological data base for the military operational environment. To meet this need, Tri-Service Toxicology arose out of the Army, Navy and Air

Force toxicology programmes, as part of Project Reliance, a congressionally mandated plan for inter-service collaboration.

Tri-Service Toxicology

The mission of Tri-Service Toxicology is to provide the Department of Defense (DoD) and other customers with timely solutions to current and anticipated toxicological problems employing an integrated approach to human health effects research. This mission is carried out with a budget of \$12 million and a work force of 130 professionals including military personnel, civilian employees of the DoD, and private contractors.

Tri-Service Toxicology has many customers within the DoD, including material developers, operational commands, health and hygiene agencies, surgeons general, etc. Through the use and development of advanced methods, innovative toxicology research is conducted for operational, environmental and occupational arenas. Performance decrement test batteries, physiologically-based pharmacokinetic modelling, *in vitro* screens and mechanistic studies, in addition to standard toxicity tests, are used to evaluate the potential hazards posed by new chemicals and materials in mission-relevant exposure situations. Tri-Service Toxicology is cultivating better partnerships with material developers to improve weapon systems performance, control product life cycle costs, improve human safety, and preserve the environment for future generations. The vision of Tri-Service Toxicology is to be an integral asset within the DoD as the centre of excellence for toxicology research, solving the challenges of today and anticipating those of the future.

Laboratory operations

Research projects in which Tri-Service Toxicology will participate are identified and priorities assigned by a Scientific Council. This Council identifies appropriate projects, selects a project leader, and assembles the research team needed to complete the project using a matrix management approach. All research activities of Tri-Service Toxicology are performed by members of seven Technology Area Groups (TAGs).

- (1) Aerosols;
- (2) Pharmacokinetics;
- (3) Pharmacodynamics;
- (4) Neurobehavioural Toxicology;
- (5) Hazard Assessment;
- (6) Analytical Chemistry; and
- (7) Pathology.

The unique character of these groups and some historical highlights are described below.

Aerosols

The Thomas Domes individually-airlocked chambers designed to provide the Air Force and Navy with hypobaric continuous exposure capabilities, are of historic interest in the field of inhalation toxicology. The original domes were constructed in 1964 to support the 'Man-in-Space' programme. For 30 years, these domes were the foundation of inhalation exposure studies on the toxicity of a number of atmospheric contaminants. They continue to provide Tri-Service Toxicology with the capability of generating extended continuous inhalation exposures of contaminants whose toxicity is not characterised sufficiently. This capability permits the accurate assessment of the hazard these contaminants represent under militarily-specific exposure conditions (eg, closed atmospheres in tanks, submarines, cockpits, etc.). A number of hydrocarbon fuels have been tested in 90-day continuous or 1-year intermittent exposures in the domes.

In addition to the Thomas Domes, a number of smaller inhalation chambers, in addition to nose-only exposure systems, are used in inhalation toxicity studies. Rodents can be exposed to particulate matter, gases or aerosols. Small chambers are used for acute tests, such as the inhalation limit test, in addition to longer-term studies lasting up to 90 days. Recently, a 90-day intermittent inhalation test was conducted for chlorotrifluoroethylene (CTFE) oligomers, a base stock for a non-flammable hydraulic fluid.

Pharmacokinetics

Toxicologists at Wright-Patterson AFB have been, and continue to be, among the leaders in the development and use of biologically-based kinetic (BBK) models. Mel Andersen, PhD, was instrumental in initiating the modeling efforts for the military, first as an

officer in the Navy and later as a civilian working for the Air Force. BBK models mathematically describe the dynamics of chemicals in the body, taking into account metabolism, permeability of membranes and partitioning of chemicals into tissues. A BBK model is developed by evaluating the biological system under consideration and identifying individual tissues of interest or groups of tissues based on similarities in blood flows and partition coefficients. Model parameters are determined by laboratory studies or obtained from literature values. Physiological parameters representative of one species can be interchanged with those of another species to extrapolate across species. Absorption, distribution, metabolism, storage and elimination of a chemical are then mathematically described for each compartment, as appropriate, and the kinetic behaviour of the chemical simulated by numerical computations on micro- or mini-computers using ACSL (Advanced Computer Simulation Language, Mitchell and Gauthier Associates Inc., Concord, MA) or Simusolv (Dow Chemical Company, Midland, MI). This approach is being applied with significant success in the risk assessment process.

In the 1980s, gas uptake chambers were developed to support BBK modelling of volatile organic chemicals. Test chemicals are injected into the closed uptake chambers containing small numbers of experimental animals. Following a distribution phase, the disappearance of the chemical from the chamber is determined by the rate of metabolism of the chemical in the experimental animals. Using a series of exposures at different concentrations of the test chemical, a toxicologist can estimate the apparent *in vivo* V_{max} and K_m of the chemical. These experimentally determined metabolic parameters can then be used in BBK models to estimate the dose of parent chemical and metabolites to target tissues.

Neurobehavioural toxicology

The effects of operational exposures to chemicals on the central and peripheral nervous system are of significant importance with respect to mission performance and long-term

health effects. Toxicologists at Wright-Patterson AFB are capable of developing comprehensive profiles of potential neurotoxicants. For example, studies of trimethylolpropane phosphate (TMPP), a potent proconvulsant produced through the pyrolysis of certain synthetic lubricants used in military ships and aircraft, include: dose-response relationships, gender susceptibility, electroencephalograph paroxysmal activity, and chemical kindling and extinguishing. In this project, concurrent biochemical studies determined the anatomical disposition and clearance of the compound after single or multiple exposures using radiolabelled TMPP. These neurotoxicity profiles provide the necessary data for establishing safety procedures under operational conditions.

Neurobehavioural end-points are also included as part of 90-day studies in order to screen chemicals for potential neurobehavioural toxicity. A 90-day study for the Army, testing a liquid gun propellant, LP1846, included evaluations of animal activity and auditory startle responses. Combining behavioural and reproductive end-points with a 90-day repeated dose study yields appropriate data for the Screening Information Data Set (SIDS). The objective of the SIDS, as originally proposed by the Organisation for Economic Cooperation and Development (OECD), is to provide a standardised initial screen of new and previously existing chemicals for risks to health and/or the environment.

Pharmacodynamics

Understanding the molecular mechanisms by which chemicals elicit their effects is important for the adequate and reliable evaluation of chemical safety. In addition, the identification of biomarkers, indicators of exposure and/or effects, can significantly improve our ability to monitor adverse effects both in the laboratory and in populations at risk. The Pharmacodynamics TAG is responsible for these issues at Tri-Service Toxicology. Support for development of this technical area has come from the Air Force Office of Scientific Research.

A number of research activities at Tri-Service Toxicology involve *in vitro*

techniques. The laboratory recently established precision-cut tissue slices for metabolic and toxicity studies as an alternative approach to mechanistic studies using whole animal exposures. In these studies an organ, such as the liver, is removed from a humanely euthanised animal, or in some cases obtained from rejected human organ transplants, and prepared for the automated tissue slicer. Slices of identical size and thickness are cut and placed in an incubation medium containing a known concentration of test chemical. After incubation, the toxicity of the compound is assessed through viability tests and by monitoring biochemical functions of the cells in the slice. Analysis of these data can be used to generate dose-response and time course data. Media and/or tissue can also be sampled and analysed to determine metabolic products. These *in vitro* data provide valuable information about the mechanism of action of new and unique chemicals of concern to the military.

In addition, the *in vitro* approach can be applied to both primary cultures of animal/human cells and transformed cell lines to examine cytotoxic effects of chemicals on specific cell types. Primary cultures of rat hepatocytes have been used to screen and rank series of related chemicals, and the data obtained have been used to make decisions about materials development. Developmental research using flow cytometry and confocal microscopy will provide new tools for investigating the mechanisms of action of relevant chemicals.

Analytical chemistry

An extensive range of analytical methods can be performed by Tri-Service Toxicology scientists. In addition to HPLC and GC instrumentation, the laboratory possesses the capabilities for mass spectrometry, FTIR spectrometry and thermogravimetric analysis. Recently the analytical chemistry group identified an artifact in the analysis of dichloroacetic acid (DCA) that results in the overestimation of the concentration of DCA in blood samples. A metabolite of trichloroethylene (TCE), DCA was previously thought to be involved in the promoting effect observed with chronic exposure to TCE. This analytical artifact may have important ramifications in the risk assessment of TCE.

Pathology

Complete histopathologic examinations of tissues obtained from both *in vivo* and *in vitro* studies are available through Army Veterinary Pathologists assigned to Tri-Service Toxicology. In addition to examination of standard paraffin- and plastic-embedded tissue sections, special stains, electron microscopic and confocal examinations are performed routinely. Both a transmission and scanning electron microscope, the latter equipped with an X-ray analysis system, are used for ultrastructural analysis of tissues and cells. A state-of-the-art computerised image analysis system is on-site to process and analyse images obtained from light and electron microscopy. It is of historical note that Tri-Service Toxicology was the first laboratory to report hyaline droplet nephropathy in male rats produced by exposure to petroleum hydrocarbons. It was later shown elsewhere that this effect is a species/sex specific phenomenon unique to male rats (the α_2 -microglobulin story) and is not a marker for human renal pathologies.

Current projects

Tri-Service Toxicology is involved in a wide range of research activities. A summary of projects currently in progress is shown in Table 1. The Trichloroethylene (TCE) Carcinogenicity Project will have a significant impact on remediation levels of TCE proposed by the US Environmental Protection Agency (EPA). The current levels for TCE were set using a paradigm that treated all carcinogens as mutagens. However, TCE is generally acknowledged to be a promoter, not a mutagen. Research into the biological effects of TCE is being conducted to support the classification of TCE as a promoter. Kinetic and dynamic models are being developed to facilitate mechanistically-based extrapolation between species. TCE metabolic parameters in rodents and man are also under investigation.

The DoD is evaluating chemical replacements for the fire extinguishants, Halon 1211 and 1301 (Table 1). The halons are ozone-depleting chemicals that must be phased out of use under the international treaty known as the Montreal Protocol. Tri-Service toxicologists are investigating

Table 1. Tri-Service Toxicology current projects.

Trichloroethylene carcinogenicity
Halon (fire extinguishant) replacement toxicity
Combustion products of lubricants
Toxicity evaluation of explosives and propellants
Environmental research initiative - predictive toxicology programme
Combustion toxicology of advanced composite materials
Toxicokinetics of dermal penetration
<i>In vitro</i> approaches to predictive toxicology
Neurotoxicity profiles of trimethylolpropane phosphate (TMPP)

the toxicity of replacement compounds being considered for fire suppression and extinguishment. Physical endurance and cognitive impairment paradigms are used to compare the relative neurotoxicity of replacements with existing agents, while cardiac sensitisation tests are conducted to compare relative cardiac toxicity.

The Environmental Research Initiative is an Air Force Office of Scientific Research (AFOSR) project. The initiative is broadly managed under the umbrella of a programme designed to understand and quantify (1) the processes that govern the dispersion and alteration, by physical, chemical or biological means, of a contaminant as it moves through the environment from its release point to a human target and (2) the toxicological impact on that target. It involves co-ordination between several laboratories to develop information that will ultimately lead to the development of numerical and analytical tools that may be used to predict the fate, transport and health risks of future materials. The initiative will also provide a validated process for determining appropriate remediation efforts for existing environmental contaminants impacting on DoD operations. Tri-Service Toxicology participates in this project through the Predictive Toxicology Program. The goals are to (1) study the link between target tissue dose and toxicity using biologically-based modelling and (2) identify mechanistic biomarkers and metabolic pathways to estimate animal/human exposure and to predict toxicity. Current efforts in the laboratory are focused on (1) the development and refinement of biologically-based response models of exposure-related biological effects (lipid peroxidation by TCE and metallothionein induction by cadmium) and (2) the collection of *in vitro* response data for multiprotein induction in a uniquely designed genetically-engineered system

that can be applied to the development and validation of a quantitative structure-activity model.

Collaborations

Extramural collaborative efforts are seen as a resource and an effective way of doing business. A number of related research projects are being conducted at universities such as Wright State University, University of Illinois (Urbana-Champaign), Indiana University-Purdue University (IUPU) and Colorado State University. These collaborative activities allow Tri-Service Toxicology to tap into academic research resources that complement the capabilities of the laboratory.

Tri-Service toxicologists work closely with the other DoD laboratories in order to help screen candidate chemicals and materials while they are still in the developmental stages and while choices can still be made between alternative chemicals. Collaborative efforts entail participation on Joint Army, Navy, NASA and Air Force committees, working groups and Integrated Product Teams, such as the Environmental Safety and Health Subcommittee. Personnel from Tri-Service Toxicology actively participate on Total Petroleum Hydrocarbon (TPH) Criteria Working Group evaluating the current TPH standard used by many states to

remediate fuel contaminated sites. Other activities of Tri-Service Toxicology include participation in the Risk Assessment Methodologies Working Group, a collaborative effort with the US EPA and Department of Energy to identify better ways to conduct risk assessments. Tri-Service Toxicology is also a member of the JP-8 plus 100 Integrated Product Team representing an alliance of industry, academia and DoD to improve the thermal stability of JP-8 jet fuel and to develop less toxic fuel system icing inhibitors. These are a few examples of how Tri-Service Toxicology maintains contact with industry and other government agencies to keep abreast of developing toxicological issues.

Conclusions

The three military components of Tri-Service Toxicology, the Army, Navy and Air Force, are committed to the two-fold mission of the laboratory: (1) to provide reliable and timely assessments of toxicological issues for the DoD through an integrated approach to human health effects research and (2) to improve the science of toxicology by developing new tools for the qualitative and quantitative prediction of adverse health effects in human populations. The process of programme development is essential in order for Tri-Service Toxicology to continue to improve, grow and better serve its customers and the operational aspects of the DoD. Although Tri-Service Toxicology is itself a relatively new entity, each of the three component services have demonstrated a long history of significant contributions to the field of toxicology. Collectively, they will continue that tradition into the future under the auspices of Tri-Service Toxicology.

TEN REVIEWS

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**TOXICOLOGY ON THE INTERNET:
A RESOURCE FOR TOXICOLOGY INFORMATION EXCHANGE**

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and ¹Geo-Centers, Inc, Wright-Patterson AFB, OH

The purpose of this poster is to acquaint the reader with the recently established Tri- Service Toxicology internet capabilities and internet usage and to provide information on how to use these resources. The Tri-Service Toxicology Consortium continually moves toward meeting the ever-increasing demands for toxicology information which can be used for human health protection and resolution of environmental issues within the DoD. The establishment of internet access and tools for information will significantly enhance our ability to meet these demands. Available resources are a World-Wide-Web Server, a Gopher Server, a File Transfer Protocol (FTP) Server and a List Server which provides world wide distribution of information about physiologically based pharmacokinetic modeling issues and data needs. A brief description of how to reach these resources over the internet, contribution subject areas, definitions of internet terms, and a demonstration of accessing these resources will be provided. Tri-Service Toxicology personnel are actively working to improve these resources, and contributions on relevant subjects are welcome. Other list groups and WAIS searchable databases germane to toxicology are being developed, and contributions from all interested parties are welcome. Our vision is to have available, searchable decision-valuable, toxicology-related expert knowledge from our research activities and to provide "one-stop" access to other internet resources of value for the practice of human health and environmental risk assessment.

Poster Abstract, 1995 Toxicology Conference. Conference on Risk Assessment Issues for Sensitive Human Populations. 25-27 April, 1995.

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dependent manner and after 1 hr treatment, cellular Ca_{2+} level reached to 140% and 160% of the control, respectively. Treatment of cells with the metals also decreased the intracellular ATP in a dose-dependent manner within 1 hr of treatment. These results suggest that the above metals may be inhibiting the energy-dependent Ca_{2+} regulating process by interacting with Ca_{2+} -ATPase pumps and this calcium overloading could, at least in part, be mediating the metal-induced cytotoxicity.

160 SUPERIOR PROTECTION AGAINST OXIDATIVE INJURY WITH HYDROPHILIC TOCOPHEROL ESTERS IN RAT HEPATOCYTES

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It has previously been demonstrated that hepatocytes incubated with tocopheryl succinate (TS) are completely protected from the toxic effects of a wide variety of toxic insults including oxidative injury. In contrast, protection was not observed with the administration of unesterified tocopherol (T) or tocopheryl acetate (TA). To test the possibility that TS cytoprotection results from the intact anionic TS molecule we examined the effect of the non-hydrolyzable derivatives of TS, tocopheroxyl butanoate and tocopheryl 3-methyl succinate, on ethyl methanesulfonate-induced cell death and lipid peroxidation. Cytoprotection was not observed with these anionic derivatives indicating that the cellular accumulation and hydrolysis of TS (releasing tocopherol) are responsible for TS protection. Next, we investigated the hypothesis that the superior protective abilities of TS result from the increased hydrophilicity of this molecule. We examined the protective capacity of other hydrophilic tocopherol derivatives, such as TS-PEG ester, tocopheryl phosphate, tocopheryl glutarate, and trolox. Each of these compounds completely protected hepatocytes from oxidative damage, suggesting that hydrophilic tocopherol derivatives are superior cytoprotective agents *in vitro*, during an acute oxidative insult (as compared to T and TA). We suggest that ability of hydrophilic tocopherol derivatives to rapidly replenish subcellular sites with a functional antioxidant is responsible for protection against oxidative injury. (Supported by NIEHS grant #05452).

161 TRICHLOROETHYLENE: FREE RADICAL STUDIES IN B6C3F1 MOUSE LIVER SLICES

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Electron paramagnetic resonance EPR/spin trapping techniques were used to test the hypothesis exposure of liver to trichloroethylene (TCE) causes increased formation of free radicals. Precision cut liver slices from B6C3F1 mice were incubated for 20 min. with TCE at headspace concentrations ranging from 0–10,000 ppm with and without the spin trap α -phenyl-tert-butyl nitroxide (PBN). PBN (10 mM) did not interfere with K^+ levels, lactic dehydrogenase, aspartamine transaminase or alanine transaminase. Free radicals were measured using a Bruker ESP300E spectrometer. A solution of 3-carbamoyl — 2,2,5,5 tetramethyl-1-lyoxy free radical was used as a standard to quantitate radicals. All data was normalized to liver wet weight. Total radicals (TR) increased linearly with increasing TCE head space concentration ($\text{TR} = 4.9\text{E} - 04[\text{TCE}] + 6.84 \times 10^{-14}$, $r = 0.99$ $P < 0.001$). EPR/spin trapping results were corroborated by conjugated diene measurements and analysis by gas chromatography.

162 TIME-COURSE OF LIPID PEROXIDATION FOLLOWING TRICHLOROETHYLENE GAVAGE IN B6C3F1 MICE

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Trichloroethylene (TCE), a common groundwater pollutant, is suggested to produce free radical species during its metabolic degradation (Steel-Goodwin et al., 1994). This may account for the evidence of lipid peroxidation, seen as lipofuscin accumulation, observed in acute TCE exposures (Elcombe et al., 1985) and in chronic studies using the major metabolite trichloroacetic acid (TCA) (Bull et al., 1990). Present time course data for TCE-induced lipid peroxidation is limited to a few hours. To extend that dataset, TCE was administered by corn oil gavage to male B6C3F1 mice 5 days a week for 8 weeks at doses of 0, 400, 800 and 1200 mg/kg. Liver homogenates were prepared from harvests at multiple time points throughout the study and analysed for thiobarbituric acid reactive substances (TBARS). TBARS rise rapidly to reach a peak

of approximately 140 nmoles per gram of liver tissue at three days. Between three and five weeks the levels drop to 50 nmole/g liver and then gradually rise to 100 nmole/g by the eighth week. In this study, the time course was independent of TCE concentration.

163 SUPEROXIDE DISMUTASE MIMETICS PROTECT AGAINST PARAQUAT-INDUCED INJURY BOTH *IN VITRO* AND *IN VIVO*

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We have employed low molecular weight superoxide dismutase mimetics based on substituted manganese meso-porphyrins to protect against a known pulmonary toxicant, paraquat, which is thought to produce injury by redox cycling with diaphorases and molecular oxygen producing the reactive superoxide radical. SOD mimetics were first tested in an *in vitro* cell culture model using calf pulmonary endothelial cells (CPA-47) and rat lung epithelial cells (L2). Cell injury was assessed by measuring release of the cytosolic enzyme lactate dehydrogenase (LDH) into the cell medium. Paraquat exposure produced a dose dependent release of LDH in both types of lung cells. The epithelial cells were more resistant than the endothelial cells to paraquat-induced injury. The SOD mimetics (MnTBAP and MnTTPyP) protected both cell types in a dose dependent manner. Neither compound appeared toxic to either cell type at the highest doses used in these studies. The protective effect of the SOD mimetics appears to be due to their dismutase activities since the zinc forms which do not possess SOD activity did not protect the cells against paraquat-induced injury. We next assessed whether MnTBAP would protect *in vivo* from a bolus dose of paraquat. Six mice were given either saline (10 ml/kg, ip) or paraquat (45 mg/kg, ip) and exposed to nebulized saline or MnTBAP (5 mg/ml) for 30 minutes twice daily. Mice were killed 48 hours after paraquat treatment. Lung injury was assessed by measuring LDH, protein and % PMN's in bronchoalveolar lavage. Paraquat increased levels of lavage LDH, protein and number of PMNs. MnTBAP did not affect any of these parameters in control animals, but significantly decreased both LDH and protein levels in the group that received paraquat. These studies suggest that these SOD mimetics may be useful agents in preventing injury associated with the generation of reactive oxygen species.

164 ALTERED EFFECTS OF THE SUPEROXIDE ANION ON ISOLATED COCHLEAR OUTER HAIR CELL SHAPE BY SUBSTITUTION OF EXTRACELLULAR SODIUM WITH N-METHYL-D-GLUCAMINE

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Exposure of the guinea pig cochlear outer hair cell (OHC) *in vitro* to reactive oxygen species generating systems induces xerotrophic or protocytotic bleb formation at the infranuclear region similar to that seen following certain ototoxicant exposures. In neurons, replacing extracellular sodium (Na^+) in the medium, with N-methyl-D-glucamine (NMDG $^+$), a relatively impermeant cation, reverses oxidative stress induced cell surface bleb formation. This study exposed OHCs to a superoxide anion (O_2^-) generating system, xanthine-xanthine oxidase (X-XO), and bathing media containing either NMDG $^+$ or Na $^+$, in order to determine the potential of preventing or reversing O_2^- induced bleb formation. OHCs were bathed with artificial perilymph containing 1.5 mM CaCl₂, 1.5 mM MgCl₂ and 10.0 mM D-glucose, in 1.0% modified Dulbecco's PBS (Na $^+$ AP; 300–310 mOsm, pH = 7.2 ± 0.1), or iso-osmotic artificial perilymph in which Na $^+$ was substituted by equimolar NMDG $^+$ (NMDG $^+$ AP). Based upon pre-exposure (30 min)/exposure (90 min)/post-exposure (60 min) conditions, groups included: a) Na $^+$ AP/ X-XO in Na $^+$ AP/ Na $^+$ AP, b) NMDG $^+$ AP/ X-XO in NMDG $^+$ AP/ NMDG $^+$ AP, c) Na $^+$ AP/ X-XO in Na $^+$ AP/ NMDG $^+$ AP and d) NMDG $^+$ AP/ X-XO in NMDG $^+$ AP/ Na $^+$ AP. Group (a) cells developed blebs during the X-XO in Na $^+$ AP exposure which lasted to the experiment end. OHCs in group (b) did not develop blebs during X-XO in NMDG $^+$ AP infusion or subsequent NMDG $^+$ AP reinstatement. Group (c) cells developed infranuclear blebs during the X-XO in Na $^+$ AP exposure, all of which disappeared upon superfusion with NMDG $^+$ AP. OHCs in group (d) did not develop blebs during X-XO in NMDG $^+$ AP infusion, but did upon Na $^+$ AP superfusion. This suggests that cell surface bleb formation in OHCs due to O_2^- generation is reversible and depends upon the presence of extracellular Na $^+$ but that the absence of Na $^+$ does not protect the plasma membrane against O_2^- induced damage.

1231

EFFICIENT TISSUE REPAIR UNDERLIES THE RESILIENCY OF POSTNATALLY DEVELOPING RATS TO CHLORDECONE + CCl₄ HEPATOTOXICITY

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Previous studies demonstrated that during early postnatal development rats are resilient to the lethal combination of chlordecone (CD) + CCl₄. The objective of this study was to further investigate the underlying mechanisms of this resiliency. Postnatally developing (20- and 45-d-old) and adult (60-d-old) male S-D rats were maintained either on normal diet (ND) or 10 ppm CD for 15 days. On day 16, rats from each dietary protocol received a single dose of CCl₄ (100 µl/kg, ip) or corn oil. Liver injury was assessed by serum enzyme (ALT & SDH) elevations as well as by histopathology during a time-course 0-96 hr. Hepatocellular regeneration was assessed by ³H-thymidine (³H-T) incorporation into hepatic nuclear DNA and proliferating cell nuclear antigen (PCNA) studies. The expressions of transforming growth factor-α (TGF-α) and proto-oncogenes (*c-fos* & *H-ras*) were measured in 20- and 60-d ND rats. In ND + CCl₄ rats, transient liver injury occurred regardless of age as indicated by ALT & SDH levels and histopathological lesions. In CD rats, CCl₄-induced toxicity progressed with time culminating in 25 and 100% mortality in 45- and 60-d rats, respectively, by 72 hr after CCl₄. Treatment of 45-d CD rats with antimitotic agent (colchicine, 1 mg/kg, ip) resulted in 75% mortality by 96 hr after CCl₄. ³H-T incorporation and PCNA studies indicate delayed and attenuated DNA synthesis, indicating unrestrained progression of liver injury leading to death of the animals. In contrast, in 20-d rats CCl₄-induced DNA synthesis was efficient and substantial, the peak being between 24 and 72 hr after CCl₄ regardless of CD pretreatment. There were 3- and 3.5-fold increases in TGF-α and *H-ras* mRNA expressions, respectively, during the maximal DNA synthesis in 20-d ND rats, whereas only 2- and 2.5-fold increases were observed in 60-d ND rats, respectively. Increased expression of *c-fos* (10-fold) was observed only in 20-d rats, 1 hr after CCl₄. These findings strongly suggest that tissue repair mechanisms play critical role in the resiliency of rats during early postnatal development. (Supported by The Burroughs Wellcome Fund and ORISE).

1232 HEPATOCYTE PROLIFERATION IN F344 RATS FOLLOWING LONG-TERM EXPOSURES TO LOW LEVELS OF A CHEMICAL MIXTURE OF GROUNDWATER CONTAMINANTS

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A six-month study was performed to investigate hepatocellular proliferation and histopathological changes in F344 rats after long-term exposures to low levels of a chemical mixture of seven groundwater contaminants. The seven chemicals used are among the most frequently detected contaminants associated with hazardous waste sites; arsenic, benzene, chloroform, chromium, lead, phenol, and trichloroethylene. Male F344 rats were exposed to this mixture, or submixtures of the organic or inorganic chemicals, via drinking water for six months. The study design included a time course experiment (i.e., 3 days, 10 days, 1, 3, and 6 months) and a dose-response experiment. Hepatocellular proliferation studies were performed by subcutaneously implanting osmotic mini-pumps to continuously deliver 5-bromo-2'-deoxyuridine for 7 days which labelled nuclei of proliferating cells. In all groups, there were no differences in weight gain, body weight, liver weight ratios, or liver-associated plasma enzymes. Light microscopic evaluation revealed no lesions related to the treatments in any animals. However, significant increases in hepatocellular labelling were observed at the 3-day, 10-day and 1-month exposure time points, after treatment with the full mixture, as well as the organic or inorganic submixtures at 1X and 10X concentrations. Proliferating hepatocytes expressed a unique labelling pattern surrounding large hepatic veins (0.5-2.0 mm), but not central veins. This did not appear to be a regenerative response due to cytotoxic mechanisms as assessed by the absence of increased plasma enzyme activity and the absence of hepatocellular lesions. Ultrastructural changes in these specific hepatocytes will be presented.

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DETECTION OF PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) IN HEPATOCYTES IN TISSUE SECTIONS AFTER ADMINISTRATION OF TRICHLORO-ETHYLENE (TCE) TO MICE

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One potential mechanism of TCE-induced mouse hepatocellular tumors is the formation of excessive active oxygen species (AOS), possibly during lipid peroxidation in hepatocytes. Target (proteins, lipids, DNA) interaction with free radicals can result in activation of signaling molecules, transcription factors and/or the induction of oxidative stress-responsive genes which are potential pathways for triggering cell proliferation, paramount to tumorigenesis. To test this hypothesis using our experimental approach, we preferred the non-invasive PCNA method over bromodeoxyuridine and H³-thymidine, both which require invasive pre-administration that could potentially generate AOS. The purpose of this study was to determine the efficacy of PCNA antibody to detect TCE-induced S phase hepatocytes as a proliferation marker. Groups of mice were orally administered water, corn oil, or TCE (1200 mg/kg) in corn oil in equal volumes (once/day, 5 days/week) for 3, 6, 14, and 21 days. Histopathology, immunohistochemistry, and cell counts by image analysis revealed that PCNA antibody is an effective method for detection of TCE-induced S phase hepatocytes in tissue sections.

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EFFECT OF PIPERONYL BUTOXIDE (PBO) ON CELL REPLICATION AND XENOBIOTIC METABOLISM IN MOUSE LIVER

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We have compared the effects of PBO (a non-genotoxic pesticide synergist) and sodium phenobarbital (NaPB) on markers of cell replication and xenobiotic metabolism in mouse liver. Male CD-1 mice were fed diets containing O(control), 10, 30, 100 and 300 mg/kg/day PBO and 0.05% NaPB (equivalent to 99 mg/kg/day) for periods of 7 and 42 days. Replicative DNA synthesis was studied by implanting 7 day osmotic pumps containing 5-bromo-2'-deoxyuridine during study days 0-7 and 35-42. Treatment with PBO (100 and/or 300 mg/kg/day) and NaPB for 7 and 42 days increased relative liver weight which was associated with, respectively, either a midzonal or a centrilobular hypertrophy. Hepatocyte Labelling Index (LI) values were increased 3.5 and 8.2 fold in mice fed 300 mg/kg/day PBO and NaPB, respectively, for 7 days. LI values were not increased in mice given 10-100 mg/kg/day PBO for 7 days or any treatment for 42 days. Treatment with NaPB for 42 days increased microsomal cytochrome P-450 content and 7-pentoxyresorufin and ethylmorphine metabolism. PBO also induced some markers of xenobiotic metabolism. These results demonstrate that PBO and NaPB induce xenobiotic metabolism in mouse liver but only produce a transient stimulation of cell replication. On a molar basis PBO is less potent than NaPB. Generally PBO produced effects only at high doses (100 and 300 mg/kg/day) at which liver nodules are formed in chronic studies, suggesting a role for enzyme induction/cell proliferation in nodule formation. (Supported by PBO Task Force II).

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UNLEADED GASOLINE AND METHYL TERTIARY BUTYL ETHER INDUCE SIMILAR SHORT TERM EFFECTS IN MOUSE LIVER

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PS-6 unleaded gasoline (UG) and methyl tertiary butyl ether (MTBE), an oxygenate added to UG, induced liver tumors selectively in female mice. Given that these mice exhibited uterine alterations, and estrogen inhibits liver tumorigenesis in mice, we propose that UG and MTBE are hepatocarcinogens secondary to their interaction with estrogen. In mouse liver PS-6 increases P450 activity and estrogen metabolism, and is mitogenic and a tumor promoter. To compare a newer formulation of UG (91-01) and similarity of responses to MTBE, we evaluated the effects of 91-01 and MTBE to PS-6. Mice were exposed to 2027 ppm PS-6, 2013 ppm 91-01 or 7813 ppm MTBE vapor for 3 or 21 days under the exposure conditions of the cancer bioassays. Liver weight increases and uterine weight decreases were seen in all treatment groups. P450 activity, assessed by 7-pentoxy-resorufin-O-dealkylase (PROD) and 7-ethoxyresorufin-O-deethylase (EROD) activities, were increased similarly in all exposed mice. In the absence of hepatotoxicity, the hepatic labeling

**RISK ASSESSMENT POLICY FOR EVALUATING REPRODUCTIVE SYSTEM
TOXICANTS AND THE IMPACT OF RESPONSES ON SENSITIVE POPULATIONS**

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ABSTRACT: Risk assessment policy for evaluating environmental chemicals for their potential to produce reproductive system failures is similar to policy for evaluating cancer-causing effects. The objective of reproductive system risk assessment is to expand on the test standards that primarily focus on fertility endpoints and birth defects by using mechanism-of-action studies and quantitative risk assessment methods. An understanding of the sensitivity of reproductive system insult between animal species and from animal models to man is critical to developing risk assessment policy and test standards.

The reproductive process is complex and involves a number of maturation and sex cell development processes. Sensitivity to insult varies throughout this process, especially during 1). the development of the conceptus, sperm and ova, 2). fertilization; 3). implantation and 4). puberty. Reproductive failure has many causes and clinical effects. Risk assessment policy is

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directed toward reducing the uncertainty associated with the cause by providing a guide to understanding how dose, duration, and characteristics of the reproductive toxicant affect the reproductive process.

KEY WORDS: Developmental toxicology; EPA risk assessment policy; Extrapolating toxicity data; Reproductive toxicology; Reproductive system risk assessment; Sperm staging

1. INTRODUCTION

Claude Bernard established the basic principles for toxicologic evaluation more than 100 years ago. They remain as valid today as when they were first written and are the foundation for safety evaluation research and risk assessment. This presentation will examine several of those principles and relate them to the policies that are shaping the current regulations which guide the risk assessment of potential reproductive toxicants.

Three of these principles are key factors in risk assessment policy development:

- 1). Physiologists must discover the laws of "vital manifestations" or physiological functions, and observation and examination are the only methods of investigation.
- 2). Toxicity to target organs is determined by establishing approaches to defining the mechanism of action of drugs and other chemicals.

3). Cause and effect relationships are established through developing an objective and a hypothesis, conducting the examination and controlling the variables.

This review presents the major reproductive risk assessment policies that are currently being used. It also discusses the "new generation" methodologies to improve the information relating to potential risk to sensitive populations; the gametes, the conceptus and the adult male and female.

2. REPRODUCTIVE SYSTEM RISK ASSESSMENT POLICY DEVELOPMENT

The science relating to the toxic insult of the reproductive system has been driven by the need to prevent exposures from reproductive and developmental toxicants to sexually active adults.

Policies have evolved primarily through a series of guidelines and regulations that rely on laboratory animal surrogate models and standardized test standards that have been developed to link the causes and clinical effects of reproductive system failures (See References in Section I).

The Food and Drug Administration (FDA) issued the original test standards in 1966. These guidelines established the requirements for regulatory approval of new drugs under development.

Segment I Reproductive Effects Studies were proposed to evaluate fertility in rats. Segment II Teratology Studies were proposed to be conducted in a rodent and non-rodent animal model to evaluate birth defects and malformations in the offspring. Segment III Perinatal and Postnatal Studies were developed to evaluate potential toxicity to the young during lactation and early development. Multigeneration reproduction studies were also recommended for some pesticides

that could enter the food chain of humans. A battery of neurobehavioral tests was established at a later date to evaluate potential developmental effects on the sensory organs and the central nervous system during pregnancy.

These tests have served the regulatory process and society by using animal surrogates as test models to avoid catastrophic toxic insult to the human reproductive system. They have not been revised since they were proposed and focus primarily on fertility endpoints of malformations, functional defects, growth retardation or death. One approach for setting acceptable levels for developmental toxicity risk has been to use safety (uncertainty) factors. From a bioassay conducted at several dose levels in both a rodent and a non-rodent animal species, a supposedly safe dose for humans is determined by dividing the no-observable-adverse-effect level (NOAEL) by a safety factor. It has been suggested that a safety factor of 100 should be used when extrapolating from animal study data to establish acceptable human exposure levels (Lehman and Fitzhugh, 1954. See Section VI). If the NOAEL is taken to be a safe dose for the experimental animals, a safety factor of 10 is suggested to allow for potentially higher sensitivities of humans compared to the experimental animals and another factor of 10 to allow for differences in sensitivities among individuals. For irreversible effects, such as death or malformation, an additional safety factor of 10 is suggested (Jackson, 1980. See Section VI). Even though the safety factor of 100 is adequate to account for interspecies and intraspecies differences in response, this does not necessarily result in a risk-free dose because the power of the experiment may not be adequate to detect subtle toxic effects (Galor, 1989. See Section VI). Although this method is not foolproof for setting acceptable levels for humans, it has provided a margin of

safety and has reduced the risk for most chemical entities that have been evaluated by using these standardized tests. Most teratological studies are capable of detecting reproductive system disease incidence of 10% or more. The reason for this increase in sensitivity in humans to reproductive system toxicants is not clearly understood but is likely due to differences in metabolism and mechanism of action of the hazardous chemical. Warning labels are required for drugs and pesticides to alert physicians and sexually active humans to avoid contact with developmental toxicants, especially during pregnancy. The primary regulatory concern today is that data gaps exist for most of the chemicals in commerce, and more than 4,000 reproductive or developmental toxicants for animals do not produce these effects in humans. About 50 human reproductive system toxicants have been reported to have caused developmental toxicity in humans (Schwetz and Harris, 1993. See Section I). The regulatory policy has been largely based on preventing or reducing exposure to the mother at or below safe levels during the sensitive periods for fetal development. Prevention of exposure to human toxicants remains to be the most effective principle for protecting the reproductive system from toxic insult (See References in Section IV).

Since the promulgation of the FDA guidelines, a number of position papers, guidelines or regulations have been written to establish policy for protecting humans from reproductive system risks. Several of these policies are discussed in this presentation and a list of policies is included.

3. DISCUSSION

New risk assessment policy for going beyond the current test battery to explore the processes by which reproductive system failures and successes occur has been promulgated (See References in Section II). This policy, along with the development of new models for expanded end points that characterize mechanisms of sex cell maturation and function, is providing scientists and regulators better means for assessing chemical risks. The U.S. Environmental Protection Agency (EPA) has developed risk assessment guidelines which were finalized in 1991 (See References in Section I, 1991). These guidelines were based on the same criteria for cancer risk assessment which are routinely used today. These include:

- 1). Hazard identification
- 2). Dose-response assessment
- 3). Exposure assessment
- 4). Risk characterization

These guidelines define reproductive toxicity and developmental toxicity and describe and discuss the endpoints that need to be evaluated in order to prevent adverse effects to the reproductive system and process. The EPA guidelines define reproductive toxicity as the occurrence of adverse effects on the reproductive system that may result from exposure to environmental agents. Toxicity may be expressed as alterations to the reproductive organs and/or the related endocrine system. The manifestation of such toxicity may include, but not be

limited to, alterations in sexual behavior, onset of puberty, fertility, gestation, parturition, lactation, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive system. Developmental toxicity is defined by the EPA as the occurrence of adverse effects on the developing organism that may result from exposure before conception, during prenatal development, or postnatally to the time of sexual maturation. Adverse developmental effects may be detected at any point in the life span of the organism. The major manifestations of developmental toxicity include death of the developing organism, structural abnormality, altered growth, and functional deficiency.

Fertility and reproductive function in both males and females are evaluated in the laboratory rat in the Segment I study. The reproductive toxicity is evaluated by dosing both sexes with at least three dose levels. Sexually mature rats are dosed through a sperm cycle and female sexually mature rats are dosed with three dose levels of the test article for 14 days prior to mating. For most chemicals, only one generation is required. The offspring are then evaluated for individual and litter effects of toxicity. The U.S. Food and Drug Administration uses multigeneration studies for food additives to evaluate chemical effects on fertility, gestation, parturition, lactation development and offspring development and reproduction. Segment II developmental toxicology studies are conducted in a rodent and a non-rodent species. Pregnant rats are exposed during the period of organogenesis during days 6-15 of gestation, and pregnant rabbits are exposed on days 6-18. The pregnant dams are terminated one day prior to delivery, and the pups are examined for viability, malformation, and growth (Manson and Kang, 1994. See Section VI). The International Harmonisation Committee Guideline, (FDA,1994. See Section I), recommends the

supplementation of the standardized tests with staging techniques and mechanism of action studies. Many of these new methods are described in the references by Heindel and Chapin. (Heindel and Chapin, 1993. Chapin and Heindel, 1993. See Section IV).

The risk assessment policy guidelines have improved the earlier test standards by stating the assumptions made in the risk assessment process and standardizing the use of qualitative and quantitative data in the hazard identification and dose-response processes. The guidelines have also helped to identify research needed for reducing uncertainties and to fill data gaps. This information is included in databases (See References in Section IV), and is used along with epidemiology facts (See references in Section III), to perform a reproductive risk assessment for potential reproductive system toxicants.

The criteria that are used for cancer risk assessment are used for the risk assessment of reproductive and developmental toxicants with the following additional assumptions:

- 1). An agent that produces an adverse reproductive effect in experimental animals will potentially pose a hazard to humans after sufficient exposure.
- 2). Reproductive effects are generally the same across species except for pregnancy outcomes.
- 3). All of the manifestations of developmental toxicity are of concern, including growth alterations, functional deficits and fetal death, in addition to structural abnormalities.

4). A threshold is generally assumed for the dose-response curve for reproductive effects.

Standardization of data collection has been the primary objective of the International Harmonisation Committee (IHC) guidelines that were published in 1994 (see Reference IHC in Section I). This document introduces the concept of "most probable option" which is interpreted as optimizing the test parameters to reflect sound scientific procedures. This includes determining the optimal treatment period for both male and female animal models and the conceptus so that exposure to the toxicant occurs during the most sensitive period of maturation and development. The testing requirements will include general screens to identify potential treatment-related effects and studies to characterize the nature, scope and/or origin of the toxic effect. The screening studies will remain essentially the same as with previous guidelines. The characterization studies include optimization of test designs for kinetic and metabolism studies in pregnant/lactating animals and male fertility assessment.

The IHC recommendations for male fertility assessment includes the requirement for dosing animals prior to mating for at least one sperm maturation cycle, and performing sperm evaluation studies in addition to histological evaluation. This is accomplished by incorporating methods to evaluate sperm motility and morphology using computer assisted techniques and the staging of spermatogenesis (See Russell, L.D., Section VI). This process can also be used for oogenesis. The ovarian follicles are all present when the female is born. They exist as primordial follicles until they are individually stimulated by hormones to develop into primary follicles, secondary follicles, early tertiary follicles and Graffian follicles. The oogenesis process involves distinct

mitosis and meiosis stages. Each of these stages can be identified by characteristic structures that demonstrate the maturation and differentiation of the ova. The ability to determine the stage that the toxic insult occurred is a primary consideration in understanding how the reproductive failure was produced. The first sensitive stage of the maturation process of the gamete dictates the expression of the reproductive failure. The reproductive process is a continuum of cell growth and function. The usual end point of a toxic insult early in the development of the gamete is death to the gamete. Since there are many millions of sperm being produced simultaneously, there must be a massive insult to a majority of the sperm. This insult is detected by a loss of motility and normal morphology. Human reproductive failure can result from only a slight reduction in the number of viable sperm, but the rat has been shown to be able to produce offspring with viable sperm counts of approximately 20% of normal. Ova can be insulted at any stage of maturation, but the most rapid development stages of meiosis when the Graffian follicle is becoming functional is usually the most sensitive period for toxic insult. The "trigger points" for cause and effect relationships can occur at any point along the maturation process. The toxic insult to the sperm can produce infertility by interfering with the locomotion process or by causing biochemical changes that interfere with fertilization. The ova may lose viability and cause infertility and early reproductive failure in animal models and premature menopause in women.

Once the stage and cell types that are affected are identified, biomarkers can be used to help develop an understanding of the mechanism of action that produced the adverse event. These biochemical markers include hormones, enzymes, DNA abducts, biochemical substrates and

metabolic pathway (cytochrome P-450) pathway changes. These changes can be detected in the plasma, or the tissues of the reproductive system (See References in Section IX). This combination of assessment of the gamete maturation process, mating behavior, fertility, pre-implantation stages of the embryo, and implantation provide data for the most probable option risk assessment.

New methods models and processes have been developed to help understand the mechanism of action in the normal and abnormal reproductive process in sensitive populations (See References in Section VIII). A number of new biomarkers are currently being used to define the reproductive process and detect alterations in function that result in reproductive failures (See References in Section V). These are driving policy development and are providing a body of information that is providing the associations between causes and effects in reproductive failure. By examining similarities and common sensitivity patterns between animal species and from animals to man, an optimum risk assessment approach can be developed for environmental toxicants that affect reproduction.

New methods for utilizing all the data from animal studies for extrapolating to man (See References in Section IV A), and incorporating statistical methods (See References in Section IX A), have contributed to our ability to assess reproductive system risk. Benchmarking, comparisons of fetal-to-adult effects (A/D Ratio), and improvements in the reference dose calculations and categorical regression procedures are all being considered for improving the value of the animal data for scaling to humans (See References in Section VI B).

Standardization of test standards and comparisons of similar endpoints are critical for making risk assessments from study to study and from chemical to chemical.

Pharmacokinetic and pharmacodynamic principles can be applied for gestational and lactational modeling using Physiologically-Based Pharmacokinetic (PBPK) procedures (See References in Section IX B).

The ultimate goal of reproductive system risk assessment policy is to provide a practical and affordable method for reducing the risk from toxicants to an acceptable level (See References in Section VII). The challenge for regulators and scientists is to reduce the variables in examining potential risk factors and to link cause and effect parameters to reflect real world scenarios.

4. CONCLUSION

This presentation has briefly reviewed the process of reproductive risk assessment policy development and how it relates to methods and test standards that are being used to generate and evaluate the data for regulatory decision making. The conceptus and the adult male and female that are attempting to produce offspring are the most susceptible human populations to reproductive system toxicants. The reduction in variables improves the process of extrapolating data from experimental animals to humans. Reducing exposure levels to an environmental chemical or group of chemicals during sensitive periods across the human population has been the objective of regulatory agency policies, especially since 1983. Dose, duration and characterization of the risk factor(s) combined with the time of the insult and mechanism of

action for reproductive failure are the primary factors in developing policies that guide reproductive system risk assessments (See References in Section VII).

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**Conference on Risk Assessment Issues
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**25-27 April 1995
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ManTech Environmental Technology, Inc.

M EASUREMENT ALGORITHM USED FOR C OMPARISON OF DERMAL MUSCULATURE CHARACTERISTICS FOR SEVERAL LABORATORY ANIMALS

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Defining anatomical characteristics that influence chemical penetration through skin can be aided by computer-based image analysis (IA). The algorithm presented was used to measure depth and cross-sectional area of dermal vessels. This algorithm was developed using an IA system (Quantimet 'Oc, Leica, Inc., Deerfield, IL) to analyze routine histologic sections stained with hematoxylin and eosin. Following vertical alignment of the tissue with the epidermal surface on the left, the background lighting intensity was determined. Next the total dermis was identified by computer-based detection of the darker staining characteristics of the epidermis. The right margin or border of the detected epidermis was the anatomical location of the basement membrane. A computer generated grid was overlaid on the digitized tissue section at low magnification resulting in rows and columns of small boxes or grid cells. This allowed for the precise determination of the bounding lines of the grid cell to an anatomical feature such as the basement membrane. The size of each individual grid cell was the size of the field of view at high magnification. When the individual grid cell was viewed at a (high) magnification to visualize capillaries, the image analysis system calculates the distance from the capillary to the grid cell border in the direction of the basement membrane and the distance from the grid cell border to the basement membrane. The sum of these two distances was the depth of the detected feature from the basement membrane. The measurement of the tissue involves manually selecting a grid cell, performing steps to measure features in that cell at a higher

magnification, returning to low magnification, and selecting the next grid cell until all cells have been evaluated. The detection of endothelial-lined structures (vessels) was based on color thresholding. After the vessels are detected, they are manually defined by keyboard entry as either an arteriole, venule, or capillary. The image analysis system measurement processor then performs the desired measurement of each individually detected feature. This method was more accurate and quicker than manual methods using a calibrated ocular device. This method can be used to quantitate regional and species differences in skin layers.



BIOLOGICAL EFFECTS OF ORAL TRICHLOROETHYLENE EXPOSURE ON MALE B6C3F1 MICE

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The Air Force has rated Trichloroethylene (TCE) in the top five chemicals of concern in their Superfund sites. The Agency for Toxic Substances and Disease Registry rates TCE among the top ten contaminants in Superfund sites nationwide. The current remediation level of 5 ppb is extremely difficult, and costly, to achieve. TCE remediation levels are based upon its carcinogenic potential, as evidenced in experiments using mice. Risk assessment is based on a paradigm developed for chemicals which cause mutations. TCE, however, is thought by most cancer researchers to fall into a category of carcinogens which act through promotion (the amplification of mutations already present). Several alternative risk assessment procedures are more appropriate for promoters. The Armstrong Laboratory Tri-Service Toxicology approach to this research opportunity is to build a strong scientific basis for the reevaluation

of TCE remediation levels. This poster summarizes the investigation of several biological endpoints related to promotion using liver tissue from B6C3F1 mice exposed to TCE by corn oil oral gavage over 60 days at dose levels of 1200, 800, 400, and 0 mg/kg/day. Correlations with exposure dose are made for thiobarbituric acid reacting substance (TBARS), proliferating cell nuclear antigen (PCNA), 8-hydroxy-2'-deoxyguanosine (8OHdG), and free radicals.



REPRODUCTIVE TOXICITY SCREEN OF 1,3,5-TRINITROBENZENE ADMINISTERED IN THE DIET OF SPRAGUE-DAWLEY RATS

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Several Army installations targeted for restoration have measurable quantities of 1,3,5-trinitrobenzene (TNB). As part of the process to develop environmental and health effects criteria, a modified Screening Information Data Set (SIDS) reproductive study was performed. Male and female Sprague-Dawley rats received diet containing approximately 300, 150, or 30 mg TNB/kg diet. Mating occurred following 14 days of treatment. All dams, one-half the males, and representative pups were maintained for a total of 90 days of treatment. No mortality occurred during the study; however, a decrease in mean body weights was noted in both sexes of high-dose rats. A dose-related effect was noted in most measurements of sperm function/activity. Sperm depletion and degeneration of the seminiferous tubules were noted histopathologically. Methemoglobinemia was a common finding in the high- and mid-dose levels of both sexes. Splenic hemosiderosis was observed in the same dose groups at necropsy. No adverse effects occurred in

mating or fertility indices. No significant treatment-related differences were noted in length of gestation, sex ratio, gestation index, or mean number of pups per litter. (Supported by DoD Contract No. F33615-90-C-0532)



MULTIPLE BIOACTIVATION PATHWAYS IN CELLULAR AND MITOCHONDRIAL TOXICITY OF TRICHLOROETHYLENE (TRI) IN LIVER AND KIDNEY OF MALE RATS

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Tri undergoes both oxidative metabolism and glutathione (GSH)-dependent conjugation by cytochrome P-450 (P-450) and GSH S-transferase, respectively. Although the liver is the primary site of biotransformation, both liver and kidney are primary target organs for Tri-induced toxicity. Metabolism of Tri by these two pathways was studied in freshly isolated hepatocytes, isolated renal cortical cells, and liver microsomes and cytosol from male Fischer 344 rats to compare the ability of liver and kidney to bioactivate Tri and to assess the role of renal metabolism in Tri-induced toxicity.

Metabolism of Tri to S-(1,2-dichlorovinyl) glutathione (DCVG), a nephrotoxic metabolite of Tri, was demonstrated in both isolated hepatocytes and renal cells, suggesting that the first bioactivation step that leads to nephrotoxicity *in vivo* occurs in both liver and kidney. Rates of DCVG formation were more than 10-fold greater in isolated hepatocytes than in kidney cells. Rates of DCVG formation in liver microsomes and cytosol incubated with Tri and GSH were similar, although total activity was greater in cytosol.

Two sensitive gas chromatography (GC)

Significance of the Dermal Route of Exposure to Risk Assessment

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The skin is a route of exposure that needs to be considered when conducting a risk assessment. It is necessary to identify the potential for dermal penetration by a chemical as well as to determine the overall importance of the dermal route of exposure as compared with inhalation or oral routes of exposure. The physical state of the chemical, vapor or liquid, the concentration, neat or dilute, and the vehicle, lipid or aqueous, is also important. Dermal risk is related to the product of the amounts of penetration and toxicity. Toxicity involves local effects on the skin itself and the potential for systemic effects. Dermal penetration is described in large part by the permeability constant. When permeability constants are not known, partition coefficients can be used to estimate a chemical's potential to permeate the skin. With these concepts in mind, a tiered approach is proposed for dermal risk assessment. A key first step is the determination of a skin-to-air or skin-to-medium partition coefficient to estimate a potential for dermal absorption. Building a physiologically-based pharmacokinetic (PBPK) model is another step in the tiered approach and is useful prior to classical *in vivo* toxicity tests. A PBPK model can be used to determine a permeability constant for a chemical as well as to show the distribution of the chemical systemically. A detailed understanding of species differences in the structure and function of the skin and how they relate to differences in penetration rates is necessary in order to extrapolate animal data from PBPK models to the human. A study is in progress to examine anatomical differences for four species.

KEY WORDS: Skin; partition coefficients; permeability constants.

1. INTRODUCTION

1.1. Background

The skin, constituting about 10% of total human body weight, acts as the major interface between the homeostatic internal environment of the body and the comparatively unregulated and potentially hostile external environment. The skin primarily functions as a protective barrier that restrains entry of chemical substances into the body. The potential for occupational or accidental skin exposure to nonvolatile and volatile chemi-

cals (both of which may penetrate the barriers of the skin) requires an experiment-based understanding of chemical absorption through the skin to adequately determine risks of such exposures.

Personnel working in an occupational environment are often exposed to a variety of chemicals. Maintenance, repair and fueling operations expose workers to engine oils, lubricants, fuels, hydraulic fluids, paints and solvents. All of these types of compounds present a potential for dermal exposure. Up to 40% of all occupational illness may involve the skin.⁽¹⁾ For some substances, cutaneous absorption is a major contributor to overall exposure.⁽²⁾ The absorbed total uptake of xylene from hand skin contact with solvent mixtures for 15 min was greater than that from inhalation over a full 8-hr shift in auto body repair shops.⁽³⁾ The dermal route was found

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to be the major contributor to total polychlorinated biphenyls body burden of transformer maintenance and repair personnel.⁽⁴⁾ Gloves are of limited protection, as permeation of chemicals through glove material is known to occur.⁽⁵⁾ Absorption of chemicals through the skin now appears to be of greater significance than previously suggested by industry or epidemiological experience.⁽⁶⁾ The dermal route of exposure may not always be the most important route, but it may often contribute significantly to total exposure. For a highly fat soluble chemical such as dibromomethane, the body burden from dermal penetration compared with inhalation was approximately 6% in one rat study.⁽⁷⁾ If respiratory protection were worn but the skin was exposed, absorption of this chemical vapor would still occur. A method to compare dermal vapor exposure to inhalation exposure at the same concentration has been described as a ratio of input functions for the contribution of each route of exposure, provided that the permeability constant, surface area of skin exposed, and alveolar ventilation rate are known or can be determined.⁽⁸⁾

Chemicals in the liquid state must also be considered because many exposure chemicals exist as a neat liquid or dissolved in a liquid medium such as water. Concentrations of pure liquid are much greater than in their vapor form. This results in greater total penetration through the skin. Even though the concentration on the skin is different between a vapor and the liquid form of a chemical, the solubility of a chemical in the skin should not be affected once the chemical enters the skin unless the liquid form of the chemical alters the skin barrier. Tsuruta⁽⁹⁾ and others have reported on the percutaneous absorption of organic solvents. Morgan *et al.*⁽¹⁰⁾ demonstrated that significant amounts of volatile organic chemicals (VOCs) can be absorbed through the skin during dermal exposure of rats to low levels of this class of chemical in aqueous solutions. Absorption to neat chemical did not appear to result in the same absorption rate as chemical in aqueous solution. Permeability constants were not determined by Morgan *et al.*,⁽¹⁰⁾ but peak blood levels after neat chemical exposure were approximately an order of magnitude greater than after chemical in aqueous solution. Estimation of the significance of dermal absorption of VOCs from aqueous solutions based on data for pure liquids may not provide an accurate assessment of actual exposure levels.

Additional studies in this area have looked at chloroform, a VOC that contaminates chlorine-treated municipal tap water.⁽¹¹⁾ Individuals are, therefore, exposed to chloroform while showering with chlorine-treated tap water. In situations where water should not be consumed

due to contamination with VOCs, individuals should also consider avoiding bathing with the water.

Dermal risk is a function of exposure penetration and toxicity. A toxic chemical that cannot penetrate the skin may be limited to local toxic effects on the skin. A chemical with a relatively low toxicity potential that readily penetrates the skin and enters circulation may have systemic effects or produce target organ toxicity. Therefore, it is necessary to know the capacity of a chemical for percutaneous absorption in order to assess its overall potential risk.

2. ESTIMATES OF DERMAL PENETRATION

Various methods have been used to measure the potential of a chemical to penetrate the skin. The permeability constant (K_p) of a chemical is a quantitative expression of the capacity of a chemical to enter and diffuse through the skin. Permeability constants are used to predict the absorption rate or flux, which is the mass of chemical absorbed per unit area of skin per unit time. The solubility of a chemical in skin (partition coefficient [PC]) is an important parameter for determining the permeability constant when the diffusion coefficient for skin is known.

Flux, or rate of penetration of a chemical across the skin, is determined by concentration at the skin surface, the surface area exposed, and solubility of chemical in the skin.^(12,13) Skin-to-air PC values for a chemical are a measure of the solubility of the chemical in skin and should correlate with the permeability constant as shown in the following equation for flux:

$$\text{Flux} = \frac{Dk_m C}{l} = k_p C$$

where Flux is mg/cm²/hr, D is the diffusion constant of the chemical in the skin (cm²/hr), k_m is the solubility or PC of the chemical in skin (unitless), C is the concentration of chemical on the surface of the skin (mg/cm³), l is the skin thickness (cm), and k_p is the permeability constant (cm/hr).

Physical and chemical properties of chemicals such as solubility are important descriptors of skin penetration.^(6,14,15) The PC for skin, a measure of the affinity of a chemical for skin tissue, is the ratio of concentrations at equilibrium between the tissue and an adjacent medium, such as air, water or other environmental vehicle. Various experimental methods have been reported in the literature for determining PC values for skin. One

method uses the octanol/water PC as a surrogate for partitioning between the skin (octanol phase) and the environment or vehicle (water phase).^(16,17) Octanol/water PC values are typically determined by shaking the test compound in a mixture containing equal parts of water and octanol. After sufficient time for equilibration to occur, the ratio of the amount of test compound in each solvent is determined.⁽¹⁶⁾ Hawkins and Reifenrath⁽¹⁸⁾ compared octanol/water PC values to the percent of applied dose of pesticides and steroid hormones after exposure *in vitro* through pig and human skin. Kasting *et al.*⁽¹⁷⁾ used octanol/water PC values in a mathematical model to estimate the flux of chemicals across the skin. Berner *et al.*⁽¹⁹⁾ used octanol/water PC values to confirm skin permeation rates for a series of chemicals prior to examining the relationship between the pKa of these chemicals and acute skin irritation. Octanol/water PC values have been used to estimate dermal flux for setting a skin notation guideline for a threshold limit value-time weighted average.⁽²⁰⁾ Although the octanol/water PC has been used extensively in estimating dermal penetration, it is an oversimplification of the process of chemical interaction with the skin. The octanol/water PC assumes that skin is homogenous with respect to octanol and that water is the environmental medium.

Surber *et al.*⁽¹⁵⁾ measured stratum corneum (SC)/water and SC/isopropyl myristate PC values. In their study, PCs were determined as a function of equilibration time, initial concentration of drug in the vehicle, delipidization of stratum corneum, and source and preparation of stratum corneum. The PCs were considered as predictors of percutaneous penetration for the purpose of conducting dermal risk assessments.⁽¹⁵⁾

3. TIERED APPROACH

A tiered approach is proposed for determining the potential hazard of a chemical for dermal risk assessment as shown in Fig. 1. This approach employs toxicity tests in an orderly sequence that can be stopped at various levels depending on the application and potential for exposure of the chemical, potential for full-scale development of the system of intended use, initial toxicity results, etc.

The first phase is conducted completely with *in vitro* tests and structure-activity comparisons. Dermal PCs are proposed as an important first step at this level. A procedure for determining skin:air PC values was developed in this laboratory and will be summarized in this paper. Exposure assessment is also an important early

component of the tiered approach. Knowing the physical form of the chemical, the expected concentration, and possible environmental medium are essential in planning the appropriate tests to conduct initially as well as throughout the tiered approach.

The second phase involves acute *in vivo* toxicity studies such as a dermal limit test. The Phase I screen is used to eliminate as many chemicals as possible in order to decrease the number of animal studies.

Another relatively early step in the tiered approach is the development of a physiologically-based pharmacokinetic (PBPK) model with a skin compartment. The use of PBPK models will also be discussed, and an example of their use is presented in this paper. Physiologically-based pharmacokinetic model development spans two levels of the tiered approach because development of a model involves *in vivo* procedures. A PBPK model could still be developed without completing all of the endpoints for a Phase II screen. Completion of a PBPK model and short-term dermal exposure studies represents the Phase III screen.

Phase IV is the screening phase for genotoxicity and carcinogenicity. Completion of this phase would provide a comprehensive hazard assessment of potential dermal risk. It is possible that *in vitro* genotoxicity testing will need to be conducted prior to the completion of earlier phases.

4. SKIN:AIR PARTITION COEFFICIENTS

The headspace method for PC determination, developed by Sato and Nakajima⁽²¹⁾ and modified by Gar-gas *et al.*,⁽²²⁾ has been used extensively in this laboratory for determining PCs of a variety of biological tissues. However, the methodology was not adequate for measuring the skin:air PC. A modification in the preparation of skin for the headspace method was developed in order to measure skin:air PC values.

Dibromomethane was used as a prototype for skin:air PCs. Male Fischer 344 (F-344) rats (Charles River Laboratories) were between 8 and 16 weeks old at the time the skin was collected for PC determination. Clipped dorsal skin was collected and cut into 1 × 0.5 cm strips. The pieces of skin were placed on the walls of scintillation vials (24.65 ml volume) without saline. Sample vials containing skin and the corresponding empty reference vials were injected with an equal concentration of chemical vapor. At equilibrium, vapor from the headspace of the sample and reference vials were measured on a gas chromatograph with a flame ionization detector.

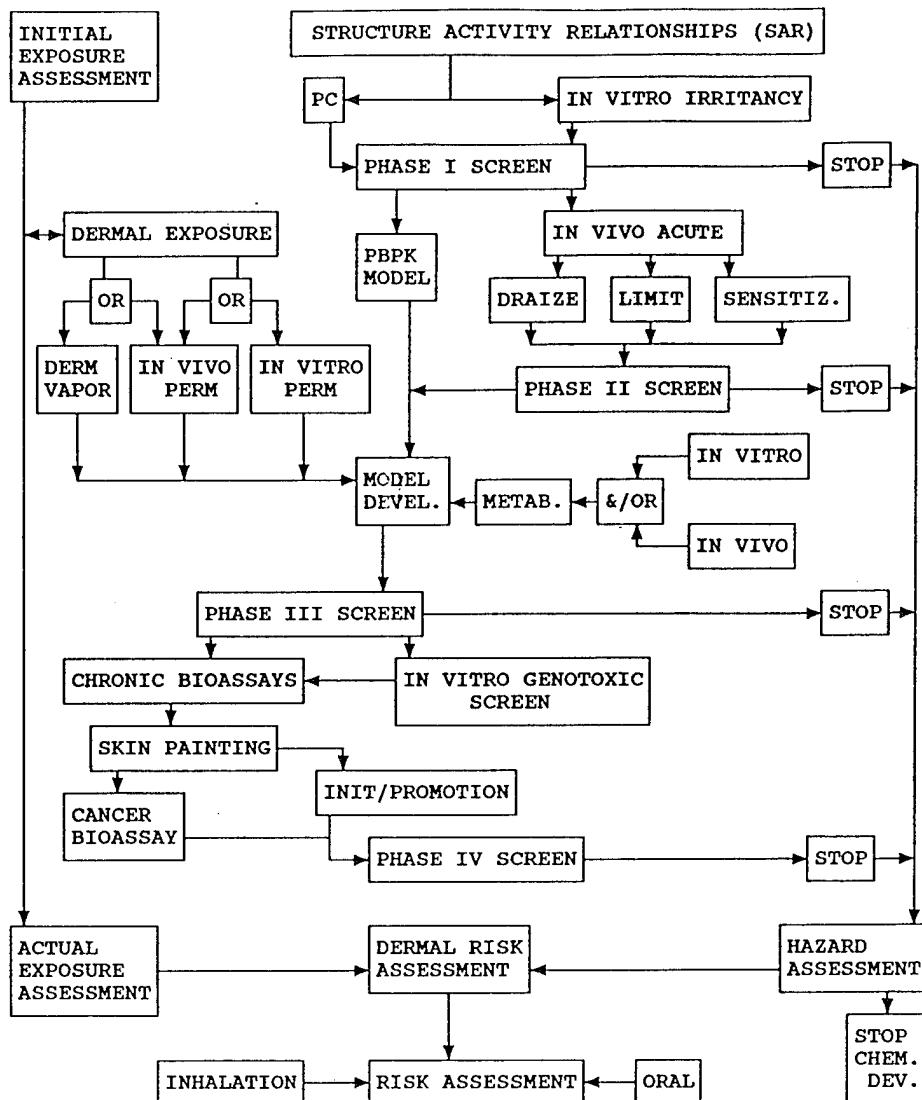


Fig. 1. Tiered approach to dermal risk assessment.

After measuring the amount of chemical as area counts from all of the reference and sample vials in a set, sample vials were compared with corresponding reference vials using the following equation modified from Gargas *et al.*⁽²²⁾:

$$PC = \frac{(reference\ vial\ area\ counts)(vial\ volume) - (skin\ sample\ area\ counts)(vial\ volume - sample\ volume)}{(skin\ sample\ area\ counts)(sample\ volume)}$$

After developing the technique for determining a skin:air PC value using dibromomethane, the procedure was used for other VOCs of interest (Table I).

The skin:air partition coefficient for dibromome-

thane and selected VOCs is shown in Table I. Approximately 19 samples were analyzed for each chemical. The most common equilibration time for this group of volatile chemicals was 4 hr. The skin:air PC values ranged from 1.9 for hexane to 91.9 for styrene. The octanol/water PC values⁽²³⁾ were compared with the corresponding skin:air PC values for 11 of the above chemicals. There was no correlation ($r^2 = 0.09$) between the octanol/water PC values and skin:air PC values (Fig. 2). Permeability constants were available for nine of the chemicals for which skin:air PC values were measured in this study.^(7,13,24) There was good correlation ($r^2 = 0.93$) between permeability constants and skin:air PC values (Fig.

Table I. Rat Skin:Air Partition Coefficients for Selected Volatile Organic Chemicals

Chemical	Skin:air PC (\pm SE)	N	Equili- bration time (hr)
Dibromomethane	68.3 \pm 3.1	10	4
Perchloroethylene	41.5 \pm 1.2	16	4
Trichloroethylene	31.8 \pm 1.5	19	4
Benzene	34.5 \pm 1.9	19	4
Hexane	1.9 \pm 0.1	18	4
Toluene	43.0 \pm 1.8	16	4
Xylene	50.4 \pm 1.7	24	2
Styrene	91.9 \pm 6.8	20	3
Methylene chloride	13.6 \pm 0.5	17	2
Carbon tetrachloride	12.4 \pm 0.6	24	4
Methyl chloroform	10.8 \pm 0.6	18	4
Halothane	10.6 \pm 0.7	17	3
Isoflurane	4.5 \pm 0.3	16	6

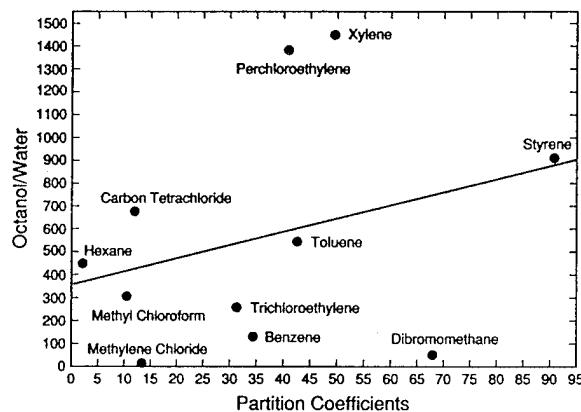


Fig. 2. Comparison of skin:air partition coefficients with octanol/water PC values.

3). When octanol/water PC values were compared directly to eight of the permeability constants (minus the octanol/water PC for isoflurane), the correlation was also poor ($r^2=0.04$). If a saline:air or water:air PC value is determined for a chemical, a skin:saline or skin:water PC value can be calculated for the chemical by dividing the skin:air PC value by the saline:air or water:air PC value. Comparison of octanol/water PC values with skin:saline PC values still resulted in a poor correlation ($r^2=0.20$).

The skin:air PC values were compared to both octanol/water PC values and permeability constants. Octanol/water PC values have been used as a qualitative measure of skin permeability.^(16-18,25,26) Skin:air PC val-

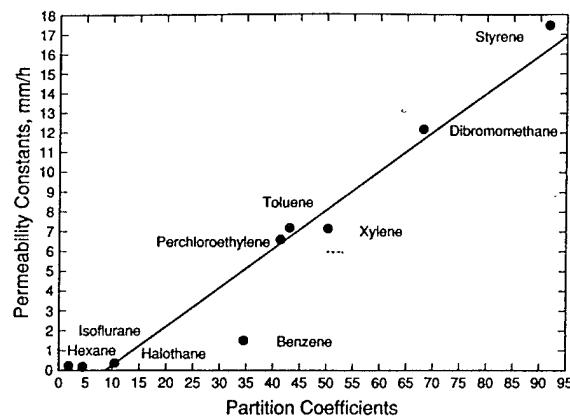


Fig. 3. Comparison of rat skin:air partition coefficients with rat permeability constants.

ues for the chemicals tested showed a correlation with permeability constants but did not show as good a correlation with octanol/water PC values. Octanol/water PC values for the VOCs examined in this study appeared to be poor indicators of the solubility of these chemicals in skin. No single bulk solvent, such as octanol, precisely mimics the solvent properties of the stratum corneum transport barrier.⁽²⁵⁾ In addition, the skin:air PC values were determined for chemicals with poor water solubility. The predictive ability of octanol/water PC values is most likely lower for these volatile chemicals because octanol/water PC values are based on water representing the vehicle or environmental medium. The data in this study suggest that skin:air PC values are a better indicator of the relative skin permeability for the volatile chemicals examined in this study. Skin:saline PC values would be representative of permeability into skin from an aqueous environmental medium. Determining a skin:air PC or skin:saline PC is proposed as an initial screen to identify the potential for skin absorption of volatile chemicals with unknown permeability constants.

5. PBPK MODELING

In addition to indicating potential permeability, skin PCs are necessary for developing the dermal compartment in a PBPK model. PBPK models mathematically describe the dynamics of chemicals in the body, including permeability of membranes and partitioning of chemicals into tissues. A PBPK model is developed by grouping various tissue types together based on similar blood flows and PCs. Each compartment has a measured physiological blood flow. Model parameters are determined from

laboratory studies or literature values and can be changed to extrapolate across species. Absorption, distribution, metabolism and elimination of a chemical are then mathematically described for each compartment which has such a process. The skin:air PC is essential for the rate equation in the dermal compartment describing the uptake of chemical from air into the skin. A skin:saline PC value is calculated, as described above, for the rate equation for uptake into skin from an aqueous medium. A PBPK model with a dermal compartment can then be utilized to determine the permeability constant for a chemical. The difference in concentration, surface area and exposure duration between the laboratory and an actual occupational situation can also be described using a PBPK model. In addition, metabolism of the chemical, which may be quantitatively or qualitatively different between experimental species and humans, can be estimated with existing methods and their impact on penetration described using a PBPK model. PBPK modeling provides the means to relate laboratory animal exposures to the human situation by extrapolating across exposure concentrations, routes of exposure and species.⁽⁸⁾ Accurate extrapolation from animal exposures to personnel in the workplace will provide the means to more quickly and efficiently set safe, but not overly restrictive, dermal exposure standards.

For a PBPK model to accurately estimate a permeability constant for a chemical in skin, a number of conditions are important. A PBPK model with a dermal compartment must be validated based on exposure for a second route of exposure, such as the inhalation route. Skin PC data should be experimentally determined for the dermal compartment. Actual dermal exposures should be conducted in order to measure the uptake of chemical into the blood. The concentration of chemical in blood after dermal exposure is then used in model simulations to estimate the permeability constant for that chemical.

Previous work with PBPK models in this laboratory has demonstrated their usefulness in extrapolation and the risk assessment process.⁽²⁷⁻³³⁾ PBPK models were developed based on the work of McDougal *et al.*,^(7,13,24) which described each of three different *in vivo* dermal exposures in rats: whole body dermal exposure to benzene vapor,⁽¹³⁾ exposure to neat benzene from a closed cell on the dorsal skin (unpublished data and Ref. 10), and exposure to saturated solutions of benzene in water also from a closed cell.⁽¹⁰⁾ The models were used to estimate the permeability constants of benzene from blood concentrations achieved during exposure to each form of the chemical. The estimated permeability constant for dermal vapor was 0.152 cm/hr, for neat benzene 0.0025 cm/hr, and for aqueous solutions 0.05 cm/hr. The phys-

ical form of the chemical and the presence of water resulted in different rates of absorption. The permeability constant for rat skin from aqueous solutions was one half the human permeability constant value used for dermal risk assessment, 0.111 cm/hr.⁽³⁴⁾ Rat skin has been reported to be more permeable than human skin by a factor of two to four,⁽⁸⁾ so the rat permeability constant was expected to be at least twice as high as the human value for benzene.

6. SPECIES DIFFERENCES IN SKIN PENETRATION

In an attempt to better understand factors affecting dermal penetration and to be able to better extrapolate between animal species and humans, a study was initiated to quantitate selected anatomical differences in skin from a number of animal species. Anatomical differences that may affect permeability include density and size of hair follicles, density of sebaceous and apocrine glands, capillary density and distance from the surface, as well as thickness of the various layers of the epidermis and dermis. Anatomical differences in skin between species will be compared to permeability constants for three model chemicals to determine possible correlations between structure and permeability. Permeability constants will be determined using PBPK models for chloropentafluorobenzene, perfluoroheptane and dichlorobenzene.

Sections of dorsal skin were collected from IAF/HA hairless and Hartley guinea pigs, fuzzy and F-344 rats, B6C3F1 and Crl:SKH1 hairless mice, and farm pigs. Pieces of skin were processed at the same time and under identical conditions for standard histopathology sections in paraffin. One set of sections was stained with hematoxylin and eosin and another set with Massons trichrome. Image analysis was conducted on sections from each strain using an image analysis system. Parameters measured were thickness (stratum corneum, stratum granulosum, viable epidermis and total epidermis); average depth and distribution of capillaries, venules and arterioles; surface area of each type of blood vessel relative to basement membrane of the epidermis; and depth and surface area of hair follicles and sebaceous glands relative to the basement membrane of the epidermis.

Exploratory data (Table II) showed that the hairless guinea pig and farm pig have the thickest epidermal layers and the F-344 rat and the mouse the thinnest epidermal layers. There was a wide range for average depth of capillaries, venules and arterioles with the hairless guinea pig and mouse having capillaries and venules closer to the epidermis and the F-344 rat having all ves-

Table II. Anatomical Parameters in Skin from Four Animal Species ($N=3$)

	Mouse		Guinea Pig		Rat		Swine farm
	SKH1	B7C3F1	Hartley	Hairless	Fuzzy	F-344	
Total epidermis	26.4 ± 1.7	13.2 ± 1.5	26.8 ± 0.7	59.6 ± 3.9	47.0 ± 3.0	20.9 ± 2.2	52.2 ± 7.2
Capillary depth	299 ± 39	421 ± 55	446 ± 149	333 ± 48	519 ± 138	803 ± 83	511 ± 13
Veinule depth	389 ± 19	536 ± 20	903 ± 218	610 ± 59	723 ± 188	970 ± 57	623 ± 87
Arteriole depth	479 ± 20	410 ± 109	602 ± 275	743 ± 298	715 ± 89	1340 ± 63	792 ± 123
Follicle volume ^a ($\times 10^{-3}$)	1.7 ± 0.4	1.2 ± 0.6	1.5 ± 0.1	2.5 ± 0.1	2.0 ± 0.5	0.5 ± 0.1	6.4 ± 2.0
Gland volume ^a ($\times 10^{-3}$)	1.4 ± 0.2	1.0 ± 0.3	0.3 ± 0.1	1.1 ± 0.2	2.4 ± 0.3	1.0 ± 0.2	5.1 ± 2.5

^a Ratio of follicle or sebaceous gland area to total area.

sels farther away from the epidermis. The farm pig had the greatest volume of hair follicles and sebaceous glands; F-344 rat the least follicular volume and Hartley guinea pig the least gland volume.

Additional skin samples will be analyzed to confirm these preliminary data. A PBPK model will be built for each of the three strains showing the widest variation in anatomical parameters. Exploratory information suggests the use of F-344 rats and Hartley and hairless guinea pigs. A better understanding of the anatomical differences of skin in animal species may lead to a better extrapolation of animal skin data to human skin.

7. SUMMARY

The dermal route is an important potential route of exposure. There is still much research to be conducted to understand the skin and its significance in risk assessment.

ACKNOWLEDGMENTS

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The animals used in this study were handled in accordance with the principles in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, National Research Council, DHHS, National Institute of Health Publication no. #86-23, 1985, and the Animal Welfare Act of 1966, as amended.

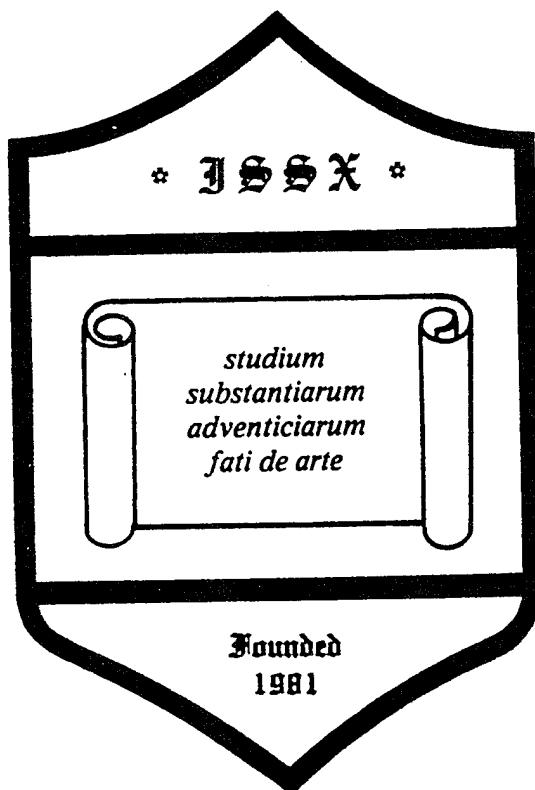
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PREPARATION OF BOVINE TESTICULAR TISSUE SLICES: EVALUATION OF 1,3,5-TRINITROBENZENE TOXICITY.

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A new animal model to evaluate male reproductive toxicity has been developed and has been used to assess the effects of xenobiotics on reproductive function. This model is noteworthy because it is a novel application of tissue slice technology, it uses tissues which normally would be discarded, and does not require the use of laboratory animals.

Recent toxicity studies with 1,3,5-trinitrobenzene (TNB), a degradation product of trinitrotoluene, have demonstrated testicular injury in rats (depletion of sperm count and degeneration of seminiferous tubules).⁽¹⁾ Human exposure to trinitrobenzene may be possible through drinking contaminated water at munitions disposal sites or through occupational exposure.

Preparation of slices of testes has been successful using bovine tissue, which is obtained from bulls used for meat processing purposes. Testicular cell relationships (interstitial cell and seminiferous tubules) are maintained and slices remain viable for more than 24 hours, as measured by histopathological examination, trypan blue exclusion, cellular enzyme (ALT, AST and LDH) and potassium leakage, and protein synthesis. Preparation of testicular slices from conventional lab animals such as the rat was not satisfactory because the tissue must be supported with agarose gel to allow slicing, and acquiring the testes necessitated sacrificing the animal. Using bull testicular slices (24 hour incubation), the level of toxicity observed for TNB was proportional to the dose. Of the parameters monitored, protein synthesis was the most sensitive indicator of toxicity; a dose/response relationship was also observed for release of intracellular potassium. As compared to controls values, protein synthesis was inhibited throughout the incubation period at a concentration of 100 µM TNB, whereas inhibition was detected only after 24 hrs at a concentration of 10 µM. Protein synthesis was completely stopped by TNB (1000 µM) and cycloheximide (100 µM). Incubation of slices with a known testicular toxicant, ethane-1,2-dimethane sulfonate (4.6 mM) as a positive control, produced a modest inhibition of protein synthesis; another known testicular toxicant, cadmium acetate (44 µM), did not change the rate of protein synthesis, as compared to controls.

Current studies are evaluating whether TNB toxicity is a result of metabolite-induced lipid peroxidation. This model offers a novel approach for studying male reproductive toxicity, reduces experimental costs and replaces the need for lab animals.

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ROLE OF NUTRITION IN THE SURVIVAL AFTER HEPATOTOXIC INJURY

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An important factor in facilitating compensatory liver regeneration after hepatic injury is the availability of adequate and appropriate nutritional support. There is regiospecificity in the primary sources of energy utilized by the hepatocytes in the periportal versus centrilobular regions. The centrilobular region derives its energy primarily by glucose oxidation while the periportal region gets its energy by fatty acid metabolism. Recent studies in our laboratory have shown that 15% glucose in drinking water for 7 days increases lethality of four structurally and mechanistically different model centrilobular hepatotoxicants (acetaminophen, thioacetamide, chloroform, and carbon tetrachloride) in male Sprague-Dawley rats ($n = 10/\text{group}$). One single injection of an ordinarily nonlethal dose of thioacetamide was lethal in glucose loaded rats and therefore was chosen for further studies. On the other hand, dietary palmitic acid (PA, 8% w/w) and L-carnitine (LC, 2 mg/ml) in drinking water protected the rats ($n = 10/\text{group}$) from an ordinarily lethal dose of thioacetamide. Serum enzyme elevations and liver histopathology revealed that actual infliction of liver injury from thioacetamide peaked between 36 to 48 hr after thioacetamide injection. Liver damage progressed thereafter in rats receiving glucose supplement, while it regressed in rats on PA-LC diet. Glucose loading did not increase the hepatic microsomal cytochromes P-450, while PA-LC dietary regimen increased P-450, suggesting lack of concordance with increased/decreased bioactivation of thioacetamide. ^{3}H -

Thymidine incorporation studies along with proliferating cell nuclear antigen (PCNA) immunohistochemical analysis of liver sections revealed inhibition of S-phase stimulation and decelerated cell cycle progression in glucose loaded rats as opposed to increased S-phase stimulation and accelerated cell cycle progression in rats on PA-LC dietary regimen. The proto-oncogenes *c-myc* and *v-Ha-ras* were expressed within 6 hr of thioacetamide administration while *v-fos* and *p53* were expressed at later time points in the rats on PA-LC dietary regimen. These findings underscore the decisive impact of nutritional supplements on the hepatobiology on one hand and the importance of tissue repair response in overcoming even massive hepatic injury on the other.

(Supported by the Burroughs Wellcome Fund)



EVALUATING THE COMBUSTION PRODUCTS OF ADVANCED COMPOSITE MATERIALS (ACM)

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The use of new synthetic materials, such as advanced composite materials (ACM), raises concerns about the potential human health risk resulting from exposure to the smoke produced by burning these materials. Methods for evaluating chemically complex combustion products are still evolving. We developed and used a novel system to evaluate the characteristics of smoke produced from controlled combustion of ACM. The combustion apparatus is a modification of the cone heater combustion module from the UPITT II method developed at the

University of Pittsburgh. Methods we employed in the evaluation of ACM combustion products include: (a) continuous plume temperature recording, (b) continuous monitoring of combustion gasses, (c) determination of mass loss rate and size distribution of the aerosol, and (d) morphologic evaluation of airborne particulate matter using light and electron microscopy, combined with computer-based image analysis. These methods significantly advance the ability to determine chemical and morphologic characteristics of the complex combustion products from burning ACM.



EFFECT OF CHRONIC PSYCHOSTIMULANT AND OPIATE USE ON SUBSEQUENT LONG-TERM APPETITIVE BEHAVIORS FOR DRUG, SEXUAL AND FOOD REWARDS: INTERACTION WITH ENVIRONMENTAL VARIABLES.

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It has been hypothesized that sensitization phenomena may play a role in long-term appetitive behaviors to seek drugs. This study assessed whether chronic pretreatment with amphetamine, cocaine, or morphine would affect subsequent amphetamine reward seeking behaviors following long-term drug relapse, and whether these effects would also generalize to more natural rewards. Since environmental variables appear to affect the development of sensitization phenomena, the drug-paired environment was additionally manipulated. Rats received 11 days treatment with amphetamine (1.0 mg/kg), cocaine (5.0 mg/kg), morphine (2.0 or 10.0 mg/kg) or saline vehicle (1.0 ml/kg) in a runway, shuttle-box or home-cage environment. Following 14 days of drug abstinence, animals were given three appetitive reward tests in counterbalanced fashion. One-half of the animals received tests of appetitive

sniffing behavior for hamburger reward on day fifteen and for sexual reward on day sixteen in an open field proximity box. On day seventeen, low dose amphetamine (1.0 mg/kg) place-preference conditioning was initiated. The remaining animals initiated amphetamine place-preference conditioning on day fifteen, followed by the appetitive sniffing tasks. The animals that received chronic psychostimulant pretreatment in the runway apparatus exhibited long-term enhanced appetitive behaviors for food, sexual and drug reward. If the animals received their chronic pretreatment in shuttle box or home-cage environments, however, appetitive behaviors for all rewards were depressed. These results suggest that repetitive use of addictive drugs may produce lasting enhancements in appetitive searching behaviors for drug reward and more natural rewards as well. Furthermore, expected response contingencies within the environment in which an organism compulsively uses these drugs can greatly enhance the development of drug-induced appetitive behaviors to attain reward.



EVALUATION OF THE DEMOGRAPHICS OF POPULATIONS LIVING NEAR TRI FACILITIES IN WEST VIRGINIA, LOUISIANA, AND MARYLAND

Susan Perlin, Ken Sexton, Sherry Selevan
U.S. Environmental Protection Agency, Washington, DC

The purpose of this study is to examine the spatial relationships among the location of Toxic Release Inventory (TRI) facilities, the total annual air emissions from these facilities, and the demographics of surrounding populations. We have used Geographic Information System (GIS)-based technology to focus on populations within small geographic areas (i.e., concentric rings of 0.5 mile radius up to 2 miles around each TRI facility), in order to test hypotheses that, on average, household income, poverty status, race/ethnicity and

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the mid-back and brought to the outside of the cage through an armored conduit. For approximately one week following surgery, the animals were infused with a heparinized saline (5 units per ml) at a low flow rate. During the test period, the animals were infused with DSW at a rate of 1.5 ml/kg/hour for 28 days. Blood samples were taken for clinical chemistry and hematology on days -4, -1, 3, 5, 7, 14, 21, and 28. Food consumption and body weight were measured weekly and urine specimens were collected overnight preceding each blood sample. A comprehensive set of tissues was collected at necropsy and processed for histopathologic examination. Under the conditions in this study, toxicity in the endpoints examined attributable to the DSW per se were not observed. Blood vessel irritation and thrombus near the end of the catheter was observed in most animals.

570 MICROTITER PLATE ASSAY (MPA) FOR LEUKOCYTE ASCORBIC ACID (AA)

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Exposure to environmental toxicants and xenobiotics often result in altered ascorbic acid (AA) status. Leukocyte (LEU) content of AA best reflects AA status; however, methods for the isolation of LEU and for AA analysis are laborious and costly. We have overcome such concerns by developing a microtiter plate assay (MPA, using a BIO-TEK Model EL340) to measure the AA-dinitrophenylhydrazine derivative (515 and 562 nm) from LEU isolated by differential sedimentation. LEU were isolated from whole blood components using Histopague 1107 and 119 (Sigma Chemical, Co). AA contribution from red blood cells was <0.15%. Incubation time for AA DNPH derivative formation was reduced to 50% with no loss in recovery (>90%). LEU AA values by our method using human subjects were in agreement with published values (0.506 mg/dl, range of 0.324–0.627 mg/dl). The AA MPA has explicit benefits over traditional methods, because it can handle small sample volumes and multiple samples simultaneously, requires the use of less hazardous reagents, and can be done on 3 ml of blood.

571 A UNIQUE, NON-INVASIVE METHOD FOR ACCURATELY IDENTIFYING RABBIT PARAVERTEBRAL INTRAMUSCULAR INJECTION SITES FOR SUBSEQUENT HISTOPATHOLOGICAL ANALYSIS

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The localized effects of drugs and vaccines administered intramuscularly is an important aspect of preclinical toxicity screening. It is often difficult to accurately locate intramuscular injection sites weeks or months after the original injection. To help overcome this difficulty, we have developed a polyethylene intramuscular injection template that is utilized during the injection procedure as well as for excision of injection sites for histopathology. The template is placed over the dorsal back area of the rabbit and aligned with the ends of the left and right caudal rib and the vertebral column. One or more injection sites are chosen and the material under study is injected to a constant depth. Excision of the injection sites requires a similar procedure whereby the animal is euthanized and the dermal tissue above the dorsal back area is removed. The template is then placed over the muscle fascia and aligned in the same manner as was used for the injection procedure. The underlying block of muscle tissue is then excised for histopathological examination. Use of the template by our lab has proven to be a significant improvement in accurately relocating intramuscular injection sites.

572 COMBINED TOXICOLOGY AND TOXOKINETIC STUDIES IN NONHUMAN PRIMATES: CHOICE AND TIMING OF SELECTED CLINICAL PATHOLOGY MEASUREMENTS

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To maximize information and minimize costs in nonhuman primate studies experimenters combine multiple research objectives into the same protocol. Inclusion of toxicokinetic/pharmacokinetic measurements into subchronic and chronic target organ toxicity studies is common. This apparently prudent approach can create interpretation problems with clinical pathology measurements that potentially overshadow meaningful objectives of the toxicologic portion of the study.

To demonstrate how this might occur, normal chimpanzees were subjected to experimental procedures simulating a combined protocol. Moderate to severe transient elevations in serum creatine phosphokinase (CPK; > 15,000 U/L), lactate dehydrogenase (LDH; >4,500 U/L), aspartate aminotransferase (AST; > 200 U/L) and alanine aminotransferase (ALT; > 100 U/L) were ob-

served. Serum CPK and LDH isoenzyme electrophoresis were helpful in eliminating apparent cardiac muscle damage as a possible contributing factor but not skeletal muscle or liver damage. Suggestions on experimental design for these combined protocols are offered.

573 DYNAMIC ORGAN CULTURE IS SUPERIOR TO MULTIWELL PLATE CULTURING SYSTEMS FOR MAINTAINING LIVER SLICE VIABILITY

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The increase in the use of tissue slices for metabolism and toxicity studies has resulted in a number of laboratories developing and applying various incubation systems and techniques to the slices. For data to be comparable from one laboratory to another a reliable and consistent incubation system must be used that will give the researcher the most optimal slice viability levels. This study compares and contrasts the dynamic organ culture system (surface culture) and the multiwell plate culture system (submersion culture). Rat liver slices were produced using the Brendel/Vitron tissue slicer under oxygenated and ice cold V-7 preservation solution. The slices (200 μ m thick) were incubated in Waymouth's medium + 10% FCS containing either NaHCO₂ or HEPES and gassed with either 95% O₂ or air and 5% CO₂. Slice viability was assessed for up to 72 hrs using ATP content, K⁺ retention, protein synthesis, ALT and LDH leakage, and MTT reduction. 7-Ethoxycoumarin metabolism was used to assess the slice's metabolic capability. The results indicated that the dynamic organ culture system maintained the rat liver slices at a higher level of viability than the multiwell plate culture system and that Waymouth's medium gassed with 95% O₂ 5% CO₂ was the best incubation condition for both systems. It is essential to optimize slice viability in order to obtain reliable and consistent scientific data which will ultimately be used to predict what will be observed in humans. (supported by NIEHS RO1-ES-05790)

574 MEASUREMENT ALGORITHM USED FOR COMPARISON OF ANATOMICAL CHARACTERISTICS OF THE SKIN FOR SEVERAL LABORATORY ANIMALS

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Defining anatomical characteristics that influence chemical penetration through the epidermis can be aided by computer-based image analysis (IA). The algorithm presented was used to measure the thickness of the stratum corneum (SC), the stratum granulosum (SG) and the remaining underlying viable epidermis (VE). This algorithm was developed using an IA system (Quantimet 570c, Leica, Inc., Deerfield, IL) and routine histologic sections stained with hematoxylin and eosin. Following vertical alignment of the tissue with the epidermal surface on the left, the initial step involved determination of the background lighting intensity. The second step was detection of the SG layer by thresholding the digitized image in a video image plane, thus storing its position and size. The third step was a similar detection of the total epidermis in an image plane. Individual SC, SG and VE measurements were obtained along a row of pixels (i.e., a chord) horizontally and each was repeated at ten pixel intervals in a vertical direction. The thickness of the SC was equal to the length of a horizontal chord between the total epidermal surface and the superficial border of the SG layer. In the loosely compacted SC, the area between cornified layers that was recognized as background lighting intensity was subtracted from the SC thickness. The thickness of the SG was measured directly from the thresholded image. The length of a horizontal chord from the deep SG border to the deepest border of the total epidermis equals the thickness of the underlying VE. This automated method allows rapid measurement of the thickness parameters in various skin samples. The method is more accurate and quicker than manual methods using a calibrated ocular device. This method can be used to quantitate regional and species differences in skin layers.

575 AN IMPROVED METHOD FOR DETERMINATION AND SEPARATION OF ORGANOCHLORINE PESTICIDES AND POLYCHLORINATED BIPHENYLS IN HUMAN BREAST ADIPOSE TISSUE

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Extensive literature exists supporting the accumulation of organochlorine compounds such as Dichloro-Diphenyl-Trichloro-ethane and Polychlorinated

Biphenyls (PCBs) in human adipose tissue. Recently, debate has surfaced concerning the link between these compounds and human breast cancer. Studies reported here detail development and validation of an improved method for determination of organochlorine pesticides and polychlorinated biphenyl isomers in human breast adipose tissue. The method involves extraction of the compounds of interest from breast adipose samples, clean-up of the residues from the extract using Florisil chromatography, separation of the PCBs from the organochlorine pesticides via silica gel chromatography, and compound identification and quantification using gas chromatography. This method yields approximately 78–94% spike recoveries and acceptable separation of the organochlorine pesticides and the PCBs. These results eliminate the possibility of interference between the two classes of compounds. This method enables distinction between organochlorine pesticides and PCBs as possible factors in breast cancer.

576 DETECTING METABOLITES IN TISSUES OF RATS EXPOSED TO ^{13}C -Labeled ACRYLAMIDE USING NMR SPECTROSCOPY

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Elucidation of pathways involved in toxicant metabolism in tissues is important in understanding the correlation between tissue susceptibility and adverse effects. Studies in our laboratory have shown significant advantages in characterization of urinary metabolites using ^{13}C -labeled toxicants and NMR spectroscopy. We are examining the feasibility of direct detection of metabolites in tissues of rodents administered ^{13}C -labeled toxicant using NMR. Male Fischer-344 rats were administered, by gavage, 100 mg/kg [1,2,3- ^{13}C]acrylamide (AM). According to previous disposition studies using ^{14}C -labels, this dosing regimen should result in tissue metabolites at concentrations feasible for NMR detection. Brain, liver, kidney, testis, blood and urine were collected at 30 min, 1 hr, 3 hr and 6 hr after administration. ^{13}C NMR spectra were acquired on tissue homogenates, plasma and urine. AM and a metabolite derived from direct conjugation of AM with glutathione were detected in the tissue homogenates, plasma and urine. Glycidamide, the epoxide of AM, and metabolites derived from direct conjugation of glycidamide with glutathione at the 2 or 3 carbon position were detected in plasma and urine. The hydrolysis product of glycidamide was detected in urine. Studies are underway to compare the method of characterizing and quantitating metabolites in tissue homogenates and water-soluble tissue extracts using solid state and solution NMR. Assignment of metabolites directly in tissues without the requirement for synthetic standards will provide a complementary approach to conventional studies.

577 COMPARATIVE EFFICACY OF THREE METHEMOGLOBIN FORMERS IN PREVENTING CYANIDE INTOXICATION

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Current therapy for cyanide (CN) intoxication consists of the intravenous administration of sodium nitrite and sodium thiosulfate. A prophylactic treatment is needed to overcome constraints of the current therapy. The present study was designed to compare the anticyanide effects of three candidate pretreatment methemoglobin (MHb) forming compounds in an anesthetized animal model using a nonlethal endpoint. The experiment was conducted in five periods, with each of nine animals receiving the vehicle control in the first period, 0.2 mg/kg p-aminopropiophenone (PAPP) in the second period, and 2.5 mg/kg WR242511AE in the third period. Four of the animals received the vehicle control in period four followed by 7 mg/kg p-aminohethophenone (PAHP) in period five, and five animals received 7 mg/kg PAHP in period four followed by the vehicle control in period five. NaCN infusion for PAPP, PAHP and WR242511AE experiments was initiated when the predicted MHb level was approximately 5 percent. Infusion was stopped ten seconds after cessation of functional breaths (respiratory arrest). Scheduled blood samples were drawn for hemoglobin (Hb), MHb, and total blood CN⁻ levels. The primary response parameters were time to respiratory arrest, percent MHb, and NaCN dose. Other data collected included heart and respiratory rates. All pretreatment regimens effectively mitigated the effects of NaCN poisoning when compared to the vehicle control ($p < 0.05$). There were no discernable differences between protection among the three compounds. These data show that the protective effect is related to the MHb level rather than specific pretreatment drug class. [Supported by USAMRDC DAMDI7-89-C-9050]

578 EVALUATION OF A NON-LETHAL ANESTHETIZED CANINE MODEL FOR EFFICACY EVALUATION OF CANDIDATE ANTICYANIDE THERAPY

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Efficacy evaluations of anticyanide therapeutic compounds historically have used lethality in unanesthetized animals as the toxic endpoint. In 1993, von Bredow *et al.* (Medical Defense Bioscience Review, 1993) reported a non-lethal, repeated-testing, anesthetized canine model. Time to respiratory arrest (RA) induced by a continuous, slow intravenous (IV) infusion of sodium cyanide (NaCN) was shown to be a consistent, well-defined endpoint in anesthetized canines. Thirty seconds after RA, an IV bolus of a methemoglobin-forming compound reversed the respiratory effects. This study was designed to determine the variability in time to RA, survivability of anesthetized dogs repeatedly infused with NaCN and treated with hydroxylamine, replicability of the procedure within an animal and between animals, and development of trends in data. Four animals were anesthetized, intubated, catheterized, and instrumented to record respiratory and heart rates. Immediately following baseline data collection, a 4 mg NaCN/mL saline solution was infused at a rate of 2 mL/min. Following a 10-second period without functional respiration, infusion was stopped. Hydroxylamine therapy was administered 30 seconds after RA. Inter-animal variability in time to RA was significant ($p = 0.04$). The average percent variability in time to RA within an animal was less than 10 percent. Baseline heart and respiratory rates were not altered significantly. Data confirm, by replicate testing, the utility of this survival model using a non-lethal endpoint. [Supported by USAMRDC DAMD17-89-C-9050]

579 COMPARISON OF ALTERNATIVE ANIMAL MODELS FOR EVALUATION OF EMETIC ACTIVITY

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The emetic potential of a consumer product is one of the parameters used in assessing its risk following accidental ingestion. Historically, the animal model used to assess this potential is the dog. Since the ferret and pigeon are sensitive to a variety of therapeutic agents for emesis, the objective of this study was to determine if they could be used as an alternative animal model for laundry and cleaning products. In the present study, varied doses of selected products were gavaged undiluted or as a solution or suspension. Control animals were dosed with water (20 mL/kg). In-life observations were made periodically during the 24 hr post-dose period. For the historical model, the ED₅₀ for inducing emesis ranged from 4 mg/kg (laundry detergent) to 4 g/kg (fabric conditioner), with emesis occurring primarily within the first hour post-dose. For ferrets, ED₅₀ values ranged from 5.6 mg/kg (laundry detergent) to \approx 3 g/kg (fabric conditioner), with emesis occurring almost exclusively within the first hour post-dose. For pigeons, ED₅₀ values ranged from 220 mg/kg (liquid laundry detergent) to \approx 13 g/kg (fabric conditioner). For dishwashing products, the pigeon ED₅₀ values were \approx 10 times higher and the mean onset time was consistently longer as compared to the ferret and the historical model, but still occurred from \approx 30 to 60 minutes post-dose. For hard surface cleaners, the relative responses were similar for all animal models (\approx 2 g/kg). These data demonstrate that both the ferret and pigeon may be appropriate animal models for assessing the emetic property of laundry and cleaning products.

580 COMBUSTION PRODUCTS OF ADVANCED COMPOSITE MATERIALS (ACM): EVOLUTION OF THE UPITT II METHOD

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The development of methods to evaluate the composition and toxicity of smoke from burning materials is an evolving science. Using new synthetic materials, such as ACM, raises concerns about the potential environmental and human health risk resulting from exposure to the chemically complex smoke produced by burning these materials. We are using methods we developed to evaluate the characteristics of smoke produced from controlled combustion of test materials. Our combustion apparatus is an evolved form of the cone heater combustion module from the UPITT II method developed at the University of Pittsburgh. The salient features of this apparatus are: (a) continuous plume temperature recording, (b) atmospheric dispersion modeling data, (c) plume gas FT-IR Spectrometer analysis, (d) TGA-FTIR of the ACM sample, and (e) sample ports to obtain soot for analytical chemistry and morphologic evaluation of particulate material using light and electron microscopy, combined with

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EFFICIENT TISSUE REPAIR UNDERLIES THE RESILIENCY OF POSTNATALLY DEVELOPING RATS TO CHLORDECONE + CCl₄ HEPATOTOXICITY

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Previous studies demonstrated that during early postnatal development rats are resilient to the lethal combination of chlordecone (CD) + CCl₄. The objective of this study was to further investigate the underlying mechanisms of this resiliency. Postnatally developing (20- and 45-d-old) and adult (60-d-old) male S-D rats were maintained either on normal diet (ND) or 10 ppm CD for 15 days. On day 16, rats from each dietary protocol received a single dose of CCl₄ (100 µl/kg, ip) or corn oil. Liver injury was assessed by serum enzyme (ALT & SDH) elevations as well as by histopathology during a time-course 0–96 hr. Hepatocellular regeneration was assessed by ³H-thymidine (³H-T) incorporation into hepatic nuclear DNA and proliferating cell nuclear antigen (PCNA) studies. The expressions of transforming growth factor-α (TGF-α) and proto-oncogenes (*c-fos* & *H-ras*) were measured in 20- and 60-d ND rats. In ND + CCl₄ rats, transient liver injury occurred regardless of age as indicated by ALT & SDH levels and histopathological lesions. In CD rats, CCl₄-induced toxicity progressed with time culminating in 25 and 100% mortality in 45- and 60-d rats, respectively, by 72 hr after CCl₄. Treatment of 45-d CD rats with antimitotic agent (colchicine, 1 mg/kg, ip) resulted in 75% mortality by 96 hr after CCl₄. ³H-T incorporation and PCNA studies indicate delayed and attenuated DNA synthesis, indicating unrestrained progression of liver injury leading to death of the animals. In contrast, in 20-d rats CCl₄-induced DNA synthesis was efficient and substantial, the peak being between 24 and 72 hr after CCl₄ regardless of CD pretreatment. There were 3- and 3.5-fold increases in TGF-α and *H-ras* mRNA expressions, respectively, during the maximal DNA synthesis in 20-d ND rats, whereas only 2- and 2.5-fold increases were observed in 60-d ND rats, respectively. Increased expression of *c-fos* (10-fold) was observed only in 20-d rats, 1 hr after CCl₄. These findings strongly suggest that tissue repair mechanisms play critical role in the resiliency of rats during early postnatal development. (Supported by The Burroughs Wellcome Fund and ORISE).

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HEPATOCYTE PROLIFERATION IN F344 RATS FOLLOWING LONG-TERM EXPOSURES TO LOW LEVELS OF A CHEMICAL MIXTURE OF GROUNDWATER CONTAMINANTS

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A six-month study was performed to investigate hepatocellular proliferation and histopathological changes in F344 rats after long-term exposures to low-levels of a chemical mixture of seven groundwater contaminants. The seven chemicals used are among the most frequently detected contaminants associated with hazardous waste sites; arsenic, benzene, chloroform, chromium, lead, phenol, and trichloroethylene. Male F344 rats were exposed to this mixture, or submixtures of the organic or inorganic chemicals, via drinking water for six months. The study design included a time course experiment (i.e., 3 days, 10 days, 1, 3, and 6 months) and a dose-response experiment. Hepatocellular proliferation studies were performed by subcutaneously implanting osmotic mini-pumps to continuously deliver 5-bromo-2'-deoxyuridine for 7 days which labelled nuclei of proliferating cells. In all groups, there were no differences in weight gain, body weight, liver weight ratios, or liver-associated plasma enzymes. Light microscopic evaluation revealed no lesions related to the treatments in any animals. However, significant increases in hepatocellular labelling were observed at the 3-day, 10-day and 1-month exposure time points, after treatment with the full mixture, as well as the organic or inorganic submixtures at 1X and 10X concentrations. Proliferating hepatocytes expressed a unique labelling pattern surrounding large hepatic veins (0.5–2.0 mm), but not central veins. This did not appear to be a regenerative response due to cytotoxic mechanisms as assessed by the absence of increased plasma enzyme activity and the absence of hepatocellular lesions. Ultrastructural changes in these specific hepatocytes will be presented.

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DETECTION OF PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) IN HEPATOCYTES IN TISSUE SECTIONS AFTER ADMINISTRATION OF TRICHLORO-ETHYLENE (TCE) TO MICE

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One potential mechanism of TCE-induced mouse hepatocellular tumors is the formation of excessive active oxygen species (AOS), possibly during lipid peroxidation in hepatocytes. Target (proteins, lipids, DNA) interaction with free radicals can result in activation of signaling molecules, transcription factors and/or the induction of oxidative stress-responsive genes which are potential pathways for triggering cell proliferation, paramount to tumorigenesis. To test this hypothesis using our experimental approach, we preferred the non-invasive PCNA method over bromodeoxyuridine and H³-thymidine, both which require invasive pre-administration that could potentially generate AOS. The purpose of this study was to determine the efficacy of PCNA antibody to detect TCE-induced S phase hepatocytes as a proliferation marker. Groups of mice were orally administered water, corn oil, or TCE (1200 mg/kg) in corn oil in equal volumes (once/day, 5 days/week) for 3, 6, 14, and 21 days. Histopathology, immunohistochemistry, and cell counts by image analysis revealed that PCNA antibody is an effective method for detection of TCE-induced S phase hepatocytes in tissue sections.

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EFFECT OF PIPERONYL BUTOXIDE (PBO) ON CELL REPLICATION AND XENOBIOTIC METABOLISM IN MOUSE LIVER

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We have compared the effects of PBO (a non-genotoxic pesticide synergist) and sodium phenobarbital (NaPB) on markers of cell replication and xenobiotic metabolism in mouse liver. Male CD-1 mice were fed diets containing O(control), 10, 30, 100 and 300 mg/kg/day PBO and 0.05% NaPB (equivalent to 99 mg/kg/day) for periods of 7 and 42 days. Replicative DNA synthesis was studied by implanting 7 day osmotic pumps containing 5-bromo-2'-deoxyuridine during study days 0–7 and 35–42. Treatment with PBO (100 and/or 300 mg/kg/day) and NaPB for 7 and 42 days increased relative liver weight which was associated with, respectively, either a midzonal or a centrilobular hypertrophy. Hepatocyte Labelling Index (LI) values were increased 3.5 and 8.2 fold in mice fed 300 mg/kg/day PBO and NaPB, respectively, for 7 days. LI values were not increased in mice given 10–100 mg/kg/day PBO for 7 days or any treatment for 42 days. Treatment with NaPB for 42 days increased microsomal cytochrome P-450 content and 7-pentoxyresorufin and ethylmorphine metabolism. PBO also induced some markers of xenobiotic metabolism. These results demonstrate that PBO and NaPB induce xenobiotic metabolism in mouse liver but only produce a transient stimulation of cell replication. On a molar basis PBO is less potent than NaPB. Generally PBO produced effects only at high doses (100 and 300 mg/kg/day) at which liver nodules are formed in chronic studies, suggesting a role for enzyme induction/cell proliferation in nodule formation. (Supported by PBO Task Force II).

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UNLEADED GASOLINE AND METHYL TERTIARY BUTYL ETHER INDUCE SIMILAR SHORT TERM EFFECTS IN MOUSE LIVER

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PS-6 unleaded gasoline (UG) and methyl tertiary butyl ether (MTBE), an oxygenate added to UG, induced liver tumors selectively in female mice. Given that these mice exhibited uterine alterations, and estrogen inhibits liver tumorigenesis in mice, we propose that UG and MTBE are hepatocarcinogens secondary to their interaction with estrogen. In mouse liver PS-6 increases P450 activity and estrogen metabolism, and is mitogenic and a tumor promoter. To compare a newer formulation of UG (91-01) and similarity of responses to MTBE, we evaluated the effects of 91-01 and MTBE to PS-6. Mice were exposed to 2027 ppm PS-6, 2013 ppm 91-01 or 7813 ppm MTBE vapor for 3 or 21 days under the exposure conditions of the cancer bioassays. Liver weight increases and uterine weight decreases were seen in all treatment groups. P450 activity, assessed by 7-pentoxy-resorufin-O-dealkylase (PROD) and 7-ethoxyresorufin-O-deethylase (EROD) activities, were increased similarly in all exposed mice. In the absence of hepatotoxicity, the hepatic labeling

1713 SAFETY EVALUATION OF AZELAIC ACID WITH PLASMA CONCENTRATIONS IN RATS AND DOGS: COMPARISON WITH HUMAN DATA

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Azelaic acid 20% cream is being developed as a topical anti-acne drug. Azelaic acid is a naturally occurring dicarboxylic acid found in cereal grain. Safety studies with azelaic acid were conducted in rats and dogs and the margin of safety was determined when compared to the human exposure. In a 6 month topical study, 20 rats/sex/group were dosed with vehicle and azelaic acid 20% cream at doses of 250 and 500 mg/kg/day applied as divided doses BID, and 1500 mg/kg/day as divided doses TID. Expressed as active compound, these doses are 50, 100, and 300 mg azelaic acid/kg/day. Azelaic acid concentrations in plasma obtained after six months of treatment were 0.126, 0.255, and 0.791 µg/ml for the 250, 500, and 1500 mg/kg/day azelaic acid treated rats, respectively. In a 6 month oral study, 6 dogs/sex/group were dosed with 0, 10, 100, and 800 mg/kg/day of azelaic acid in capsules as divided doses BID. These doses produced mean peak plasma concentrations of 2.19, 20.8, and 135 µg/ml, respectively, at the end of the study. In humans the intended dose is approximately 2 to 5 mg azelaic acid/kg/day. 12 healthy volunteers were treated with 20% azelaic acid cream topically for 7 days at a dose of 2.5 mg azelaic acid/kg. The baseline plasma concentration of azelaic acid in this study was 0.052 µg/ml, whereas after 7 days of dosing the mean peak plasma concentration was 0.136 µg/ml. In another human study, 56 patients with mild to moderate acne were treated with 20% azelaic acid cream applied topically twice daily for 12 weeks at a dose of 3 mg azelaic acid/kg/day. After 12 weeks of treatment, the mean plasma concentration of azelaic acid, 0.090 µg/ml was not significantly different than the baseline, 0.084 µg/ml. In the rat topical study, the plasma concentrations were 6 times higher than the human topical study, and in the dog oral study, the plasma concentrations were 1000 times that seen in the human study. These data show that azelaic acid topical cream is very well tolerated and also confirm the excellent safety profile of azelaic acid.

1714 TWENTY-EIGHT DAY REPEATED CUTANEOUS DOSE TOXICITY STUDY IN FISCHER 344 RATS USING GLUTARALDEHYDE (GA)

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Fischer 344 rats (15/sex/group) were treated by occluded cutaneous application with 2.5, 5.0, or 7.5% aqueous solutions of glutaraldehyde (CAS No. 111-30-8) at a dose volume of 2 ml/kg/day (50, 100 and 150 mg/kg/day) for 20 applications over 28 days. Controls were treated with distilled water. Five rats/sex (control and 7.5% groups) were held for a 4-week recovery period. Treatment-related clinical signs of skin irritation included barely perceptible erythema, color change, exfoliation, excoriation, crust and skin necrosis, which resolved in the 7.5% group after 4-weeks recovery. Reduced food consumption, body weight and body weight gains were noted in the 7.5% group during the dosing period. Treatment-related increases in reticulocyte and platelet counts observed in females were attributed to skin irritation and necrosis. Males had dose-related increases in BUN, considered to be related to skin irritation effects. Dose-related increases in absolute and relative adrenal weights observed in males and females are believed to be due to stress from repeated application of an irritant material to the skin. Microscopic evaluation of the skin revealed treatment related acanthosis, dermatitis, hyperkeratosis (females), epidermitis and dermal fibrosis (males) of the superficial layers. Dermal fibrosis persisted in these animals after 4-weeks of recovery. Under the conditions of this study, the clinical findings were considered to be secondary to localized cutaneous irritation, with no indication of systemic toxicity. A no-observed-effect level (NOEL) was not determined in this study, although the effects were minimal at 50 mg/kg/day.

1715 DERMAL ABSORPTION KINETICS OF LIQUID CHLOROPENTAFLUOROBENZENE (CPFB) AND 1,2-DICHLOROBENZENE (DCB) IN RATS AND GUINEA PIGS

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The potential for occupational or accidental skin exposure to chemicals requires a full understanding of chemical absorption through the skin. Two model chemicals (CPFB and DCB) were chosen to provide a range of water & lipid solubilities for *in vivo* study. To determine whether species differences in dermal absorption are due to physical and physiological dissimilarities, Male F-344 rats and Hartley guinea pigs were chosen to provide different characteristics in the skin. 24 hrs prior to exposure each animal was fitted with a jugular

cannula and the glass cell was attached to the animal's back. All the animals were exposed to pure liquid CPFB or DCB for up to 8 hrs in a 3.14 cm², septum sealed glass cell. Blood was serially drawn and analyzed for these chemicals. The blood concentration of the absorbed chemicals increased rapidly during the first ½ up to 2 hrs of exposure; it reached the maximum value and stayed constant up to 8 hrs. Additionally, in each species with each chemical, a dermal permeability constant was estimated with a PB-PK model. These studies suggest that anatomical species differences are important to understand in skin penetration.

1716 SKIN ABSORPTION OF FORMULATED 2,4-D, DIAZINON, AND DDT IN RATS, GUINEA PIGS AND HUMANS

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Percutaneous absorption of pesticides is a major route of occupational exposure to these compounds and accurate models of the absorption process are needed in order to conduct hazard/risk assessments. *In vitro* skin absorption assays have previously been reported to provide good models for nonformulated pesticides dissolved in acetone and applied to the skin, however the effect of the pesticide formulation constituents has not been well-detailed. The present study examines the *in vitro* skin absorption of two commercial formulations of 2,4-D, two formulations of Diazinon, and three formulation concentrations of DDT when applied to rat, hairless guinea pig and human skin. The *in vivo* skin absorption of these formulations was also tested in rats. The *in vitro* rat assays overestimated *in vivo* dermal absorption of 2,4-D and Diazinon but underestimated *in vivo* skin absorption of DDT. There was no apparent effect of the pesticide concentration (% active ingredient in the formulation) on the total amount of pesticide absorbed. A marked enhancement of pesticide skin absorption was observed *in vitro* subsequent to a soapy water wash of the skin conducted at 24 hours postexposure. This 'wash-in' effect may have important toxicological ramifications for hazard/risk assessments.

1717 TERTIARY BUTYL HYDROPEROXIDE TOXICITY AS A MODEL OF OXIDATIVE STRESS IN PRIMARY NEONATAL RAT KERATINOCYTE CULTURES

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Tertiary butyl hydroperoxide (tBHP) has been used a model compound for inducing oxidative stress in many different cell types. The purpose of this investigation was to establish a model of oxidative stress in primary neonatal rat keratinocyte cultures. Primary cultures of rat keratinocytes were established from 2-3-day-old neonatal Sprague-Dawley rat pups. After 3 days in culture, the cells were exposed for 1 to 4 hr to tBHP in concentrations ranging from 0.001 to 2.0 mM. In addition, cells were exposed for 1 to 4 hr to diamide, a glutathione depleting agent, in concentrations ranging from 25 to 2000 µM. Toxicity was assessed by measurement of propidium iodide uptake using a fluorescent plate reader. After 2, 3, and 4 hr exposure to 0.5 mM and higher concentrations of tBHP, a significant increase in propidium iodide uptake was shown when compared to untreated control keratinocyte cultures. Diamide, at exposure concentrations of 400 µM and above, significantly increased propidium iodide uptake after 2, 3, and 4 hr of exposure. When keratinocytes were simultaneously exposed to various concentrations of tBHP and 100 µM diamide, there was no significant change in the tBHP dose-response curve through 4 hr of exposure. The results indicate that tBHP may be used as a model compound for inducing oxidative stress in primary keratinocyte cultures. This model will be valuable in understanding the role of oxidative stress in keratinocyte growth, differentiation, and senescence. (Supported by a grant from R. J. Reynolds).

1718 PERCUTANEOUS ABSORPTION OF TRINITROBENZENE. ANIMAL MODELS FOR HUMAN SKIN

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The percutaneous absorption of trinitrobenzene (TNB), a potentially toxic chemical used in munitions, was studied in viable hairless guinea pig (HGP), Fischer 344 rat, and human skin. Skin was dermatomed and assembled in flow-through diffusion cells followed by TNB application in either an acetone or a water vehicle. Skin absorption was expressed as the percent of applied dose absorbed (skin and receptor fluid) in 24 hr. Rapid absorption of TNB by rodent skin was obtained with both vehicles. For HGP skin, TNB absorption was

INHALATION TOXICITY OF VAPOR PHASE TRICRESYL PHOSPHATE

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ABSTRACT

Vapor phase lubrication is a relatively new concept that shows excellent potential for high temperature applications. To generate a vapor phase lubricant, conventional liquid lubricants or lubricant anti-wear additives are heated above the boiling point of the fluid and the resulting vapors are transported to a hot bearing surface. To date, the fluid which has received the most extensive evaluation is tricresyl phosphate (TCP). When heated to the vapor state TCP is thermally decomposed. It may undergo repolymerization and possibly other chemical changes, although very little is known about the exact nature of this process.

To evaluate vapor phase triaryl phosphates for neurotoxicity, 4-hr inhalation limit tests were performed on male rats exposed to a nominal vapor concentration of 5 mg/L in air. Two days after vapor exposure, the rats were examined for NTE activity. Untreated and triorthocresyl phosphate (TOCP) treated rats were used as negative and positive controls, respectively. Results showed a statistically significant difference in NTE activity between control rats and those treated with vapor phase lubricants.

INTRODUCTION

TCP is known to have a neurotoxic ortho-isomer, triorthocresyl phosphate (TOCP), that is normally minimized in the manufacturing process. However, when TCP is used as a vapor phase lubricant, chemical reactions occur and there is concern that additional amounts of the ortho-isomer or other toxic phosphate compounds may form. Some triaryl phosphates such as TOCP are known esterase inhibitors and have been found to cause delayed neurotoxic effects in humans¹. A single exposure to a neurotoxic organophosphorous (OP) compound can produce damage to nerves after a delay of 8 to 10 days¹. This condition is known as organophosphorus-induced delayed neuropathy (OPIDN), and is characterized by axonal degeneration. OPs that cause axonal pathology interact with the enzyme neurotoxic esterase (NTE). Changes in the activity of the enzyme neurotoxic esterase (NTE) is the initial step in the delayed neurotoxicity response. This occurs within hours of exposure to a neurotoxic chemical², and changes in NTE activity can be measured in brain, spinal cord, and other nerve tissue.

Rodents have been thought to be relatively insensitive to the clinical effects of TOCP¹. However, recent studies indicate that if morphological and NTE activity assays rather than locomotor or behavioral endpoints are used, the rat may be a more sensitive indicator of OP induced delayed neuropathy³.

To evaluate vapor phase triaryl phosphates for neurotoxicity, 4-hr inhalation limit tests were performed on male rats exposed to a nominal vapor concentration of 5 mg/L in air. Two days after vapor exposure, the rats were examined for NTE activity. Untreated and triorthocresyl phosphate (TOCP) treated rats were used as negative and positive controls, respectively.

MATERIALS AND METHODS

MATERIALS

The synthetic and natural lubricants were supplied by the US Air Force Wright Laboratory, Aero Propulsion and Power Directorate, Fuels and Lubrication Division, Lubrication Branch (WL/POSL). The test compounds were a natural (Ashland) and synthetic (Durad 125) tricresyl phosphate and a synthetic (Durad 620B) tert-butylated triphenyl phosphate.

Distribution approved for public release; distribution unlimited

*This work was performed under Contract No. N00014-95-D-0048

METHODS

Test animals and treatment

Male Long-Evans rats of at least 6 months of age (375-450 g) were obtained from Charles River Breeding Laboratories, Raleigh, NC and maintained on Purina #5002 lab chow *ad libitum*. Upon arrival to the laboratory the animals were acclimated to the laboratory environment for 2-weeks prior to exposure.

A series of inhalation limit tests was performed on male rats with lubricant vapors generated by test equipment supplied by the sponsor (WL/POSL). Each test consisted of a 4-hour exposure to a nominal vapor concentration of 5mg/L, which is considered the upper "limit" for a realistic exposure. The animals were placed in holders designed for nose-only exposures to the lubricant vapors to insure the animal breathed the generated vapor concentration.

TOCP (tri-o-tolyl phosphate, >99% pure, Eastman Kodak Company Rochester, New York) was used as a positive control for the target organ effect being measured in this study, neurotoxic esterase activity. The TOCP was administered by oral gavage. Because TOCP can produce dangerous cholinergic side effects at the dose level used in this study (i.e., 1.193 g/kg), the animals were pretreated 10 min. prior to oral dosing, and 4 hours post dosing with atropine sulfate (7.5 mg/kg, subcutaneous) to protect against these side effects.

A third group of animals breathed room air only. Since they received no vapor exposure they served as negative controls for the brain NTE assay.

Vapor Generation

The vapor generation system consisted of a syringe pump connected to a stainless steel tube that was heated to 650° C by an electric furnace to vaporize the lubricant fluid (see Figure 1). Vapor condensation was prevented by keeping the transfer line heated by use of an oven.

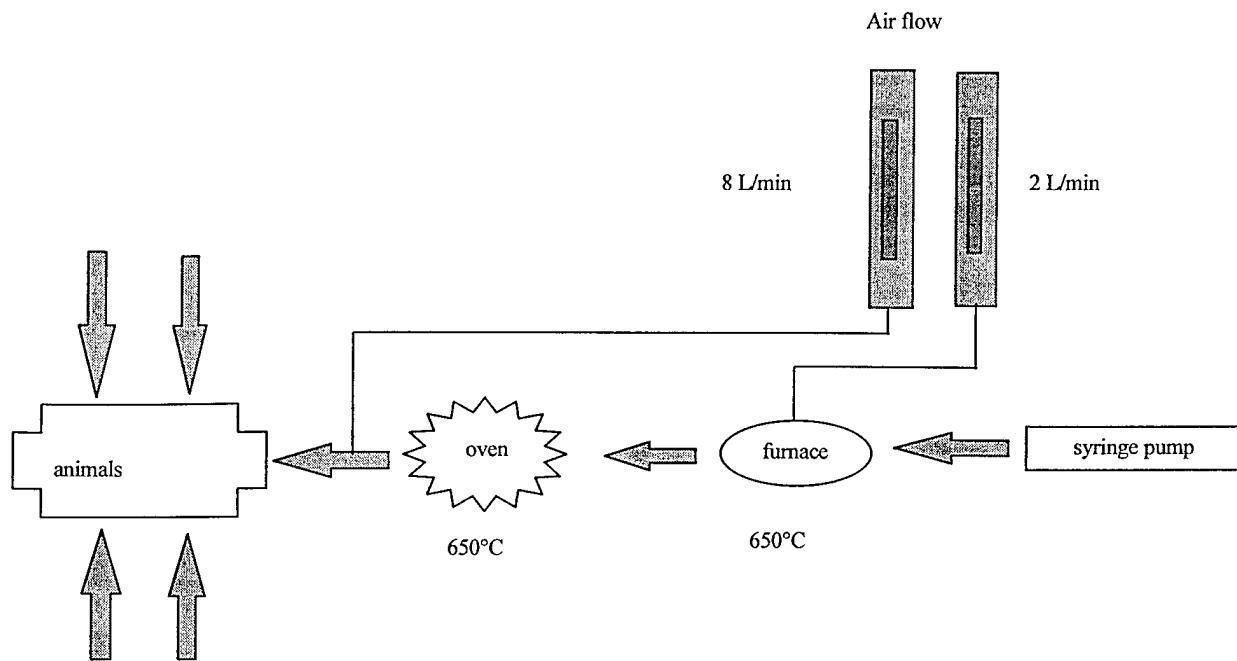
The test compound was fed into the tube with the syringe pump at a rate of 3.3 mL/hour. The density of the fluid was used to convert flow rate from a volumetric flow rate to a mass flow rate, which was used to calculate chamber concentration according to the following formula:

$$\frac{\text{DENSITY (g/ml)} \times \text{INJECTION RATE (ml/hr)}}{60 \text{ min./hr} \times \text{total air flow (i.e., 10.0 L/min.)}} = \frac{\text{g/min.}}{10\text{L/min.}} = \text{mg/L}$$

For example, the 0.0618 g/min. mass flow rate for Durad 620B was initially mixed with a carrier air flow of 2.0 L/min. This concentrated vapor flow was further diluted with an additional 8.0 L/min. air flow to provide a nominal chamber concentration of 6.18 mg/L. This is the concentration to which the animals were exposed.

An additional benefit provided by the second air stream was temperature control. During the experiment the vapor generator and transfer line temperature was maintained at 650°C; however, at no time did the animal exposure chamber exceed 30°C. This was largely a result of the addition of dilution air to the concentrated vapor immediately before entry to the animal exposure chamber.

Figure 1. Vapor Generation and Animal Exposure System.



NTE Analysis

Two days after exposure, whole brains and the spinal cord including the C-4 region were surgically removed from vapor exposed, TOCP treated (positive control), and untreated control rats. The brains were weighed and 1:150 homogenates of brain tissue in 50 uM tris: 0.02 mM EDTA, pH 8.0 buffer were prepared.

NTE activity was measured using a modification of the colorimetric assay of Johnson⁴. 50ul aliquots of brain homogenate were incubated with and without 25 ul of 12.7 uM mipafox and 100 uM paraoxon in microliter wells. After 20 min. incubation, 50 ul of 11.19 uM phenylvalerate was added as the substrate and an additional incubation period of 15 min. was provided for the reaction. The reaction was terminated by the addition of 50 ul of cocktail containing 0.008% 4-amino antipyrine, 0.08% potassium ferricyanide and 2% sodium dodecylsulfate. Condensation of phenol with 4-amino antipyrine in the presence of the oxidant potassium ferricyanide caused an orange color to develop. The intensity of the orange color was measured at 510 nm on a microplate spectrophotometer. Substrate and tissue blanks were included for each assay. NTE activity was calculated on the basis of the difference in the absorbance between the incubated samples with and without mipafox in the presence of paraoxon. NTE activity was expressed as nanomoles of phenol formed per minute per milligram brain protein.

NTE was calculated on the basis of difference in the absorbance between the incubates with and without mipafox in the presence of paraoxon. Paraoxon is used to inhibit acetylcholinesterase activity in brain, in order to measure NTE enzyme activity. The enzyme activity is calculated based on the phenol standard curve and expressed as nmol of phenol formed/15 min./mg protein.

RESULTS AND DISCUSSION

RESULTS

Analysis of NTE activity showed a statistically significant difference between negative control rats and those treated with vapor phase lubricants or orally administered TOCP (positive control). The percent NTE inhibition for each compound tested is presented in the appendix along with body weight data, and is summarized in Table 1, below. It can be seen from Table 1 that exposure to all triaryl phosphate vapor phase lubricants resulted in NTE inhibition. Durad 620B produced the least effect, with a 34.7% inhibition of NTE activity. Durad 125 produced the greatest NTE inhibition (49.3%).

Table 1. Neurotoxic Esterase Activity (in nM of phenol/mg of protein/15 minutes) and Percent NTE Inhibition from control levels.

Compound	Vapor Conc. (mg/L)	NTE activity mean (std.dev.)	Percent inhibition from control
Ashland (natural TCP)	6.4	12.130 (1.009)	43.5%
Durad 125 (synthetic TCP)	6.4-6.5	12.297 (1.244)	49.3%
Durad 620B	6.2	16.058 (0.926)	34.7%
TOCP (positive controls)	N/A	<3.3	>86%
Untreated (negative control)	N/A	>23	N/A

DISCUSSION

The level of NTE inhibition generally accepted to be predictive of OPIDN in hens is 70%¹; however, hens were notably impaired three weeks after administration of OPIDN-inducing compounds when spinal cord NTE was inhibited more than 40%⁵. Additionally, NTE inhibition as low as 31% has been shown to be associated with development of severe spinal cord damage in rats³ (see Table 2, below). Thus, the threshold for OPIDN may well be below a 70% inhibition of NTE.

Table 2. Relationship between mean NTE inhibition in the brain and severe spinal cord damage in Long-Evans rats exposed to TOCP³.

Inhibition of Brain NTE activity (% of control values)	Severe Spinal Cord Damage (% of group)
77-99%	100%
61-71%	90%
51-63%	15%
31-41%	7.5%
15-21%	0

Recently completed studies with a mixed t-butylphenyl phosphate compound administered by oral gavage did not inhibit NTE, alter motor activity, or produce neuropathology characteristic of delayed neurotoxicity at a single doses of 2 g/kg in hens⁶ or in rats (personal communication with Mr. D. Placek, FMC Corporation). Our data show that exposure to these lubricants in the vapor phase inhibits NTE. Thus, the process of vaporization is causing a change in the compound, resulting in the potential to produce neurotoxicity. Therefore, caution must be used when working with triaryl phosphate vapor phase lubricants.

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COMBUSTION PRODUCTS OF ADVANCED COMPOSITE MATERIALS (ACM): EVOLUTION OF THE UPITT II METHOD

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The development of methods to evaluate the composition and toxicity of smoke from burning materials is an evolving science. Using new synthetic materials, such as ACM, raises concerns about the potential environmental and human health risk resulting from exposure to the chemically complex smoke produced by burning these materials. We are using methods we developed to evaluate the characteristics of smoke produced from controlled combustion of test materials. Our combustion apparatus is an evolved form of the cone heater combustion module from the UPITT II method developed at the University of Pittsburgh. The salient features of this apparatus are: (a) continuous plume temperature recording, (b) atmospheric dispersion modeling data, (c) plume gas FT-IR Spectrometer analysis, (d) TGA-FTIR of the ACM sample, and (e) sample ports to obtain soot for analytical chemistry and morphologic evaluation of particulate material using light and electron microscopy, combined with

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computer-based image analysis. Endpoints obtained by this method include: FT-IR spectroscopic evolution of CO, CO₂, and H₂O; IR spectroscopy of cold trapped compounds; GC separation and mass spectroscopy identification of organic compounds extracted from soot; emission rate and size distribution of the aerosol. This testing method significantly advances the ability to determine chemical and morphologic characteristics of the combustion products.

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STOCHASTIC MODELS FOR CELL SIGNALING AND TOXIC EFFECTS ON CELLS

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December 1994

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Prepared for:

Naval Medical Research Institute/Toxicology Detachment
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STOCHASTIC MODELS FOR CELL SIGNALING AND TOXIC EFFECTS ON CELLS

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Abstract

This paper presents generic models for the effect of a chemical toxin on cells forming the tissue of an organ. The models are illustrative, not specific to organ or toxin. Interactive response of within-tissue toxin and cells is modeled: cell capability to modify (metabolize or bind) toxin is represented, as is the alteration of that capability by toxin presence. Both processes are represented in the context of simple versions of the cell cycle.

Toxin-cell interaction is explicitly represented in terms of inter-cell chemical signaling that encourages replacement or repair of cells. The impact of cell death by apoptosis and necrosis is represented in terms of within-tissue toxin concentration. The model is explicitly stochastic, representing inter-cell and toxin input and within-organ concentration in terms of diffusion approximations. Explicit mathematical discussion is given of dose-response function behavior at low doses.

1. Introduction

Application of laboratory toxicology data to environmental and human problems of risk assessment almost always requires extrapolation of the data from the experimentally-used dose regimen to the exposure conditions of practical concern, and from the animal species tested to the species of concern (usually man). This extrapolation and estimation process is known as *chemical risk assessment*. The risk assessment process has undergone considerable evolution, moving from a qualitative basis for decision making to an increasingly quantitative basis, and from the use of default assumptions to the application of mechanistic mathematical models as tools upon which to base decisions. In the context of determining safe human exposure limits to potentially toxic chemicals, there are two sub-tasks to be accomplished: estimation of low-dose risk in animals, and the subsequent conversion of animal risk estimates to human risk estimates. An authoritative survey of many of the statistical issues and opportunities is given by Krewski and Franklin (1991).

Extrapolation to low-dose effect, the first sub-task, can be accomplished either by assuming that biological response varies in a specified mathematical manner (e.g. probit, logit) with organ host exposure, or by using physiologically-based pharmacokinetic (PBPK) models to relate organ dose to host exposure; cf. Krewski and Franklin (1991), chapters 8 and 9; M. Andersen, H. Clewell Jr., and C. Travis have been prominent in this research area. In the latter approach, multi-compartment physiological models are formulated using actual tissue volumes from the experimental and target species and actual perfusion rates to provide for chemical transport between the compartments. Thus the pharmacokinetics of high to low-dose extrapolation become amenable to calculation, and external measures of external dose or exposure can be translated to concentrations in the

target organ (internal dose). Target-organ chemical concentration may be translated into estimates of risk if a suitable biologically acceptable mathematical model can be used to relate chemical presence in the organ to harmful outcome. Such models, called pharmacodynamic, exist for carcinogens, in the form of the widely-used linearized multistage approximation for cancer dose/response, and multistage clonal expansion models; cf. Moolgavkar (1988). These models are based on the general concept that chemical alteration of the cellular genes may give rise to permanent, heritable changes in the genetic information stored in the cell nucleus, and lead to phenotypic changes in the altered cells that ultimately cause the formation of malignant tumors. Simulation is sometimes used to study stochastic models of carcinogenesis for large numbers of cells; (cf. Bois (1992)).

Unfortunately, analogous dose/response models are not widely available for toxic responses other than carcinogenesis. The mathematical expression of even the relatively simple concept of genetic alteration leading to cancer involves significant simplification of biological reality, and significant mathematical complexity. This state of affairs is exacerbated when one attempts to describe the interactions leading to loss of cell function and cell death in tissues of a whole animal. The multi-layered control and response systems present in an intact living animal are poorly understood and thus have not yet been adequately modeled. Nonetheless, these control mechanisms defend against the majority of toxic effects observed as a result of chemical exposure, and their failure represents much of what is observed as the expression of toxicity.

This paper describes initial formulations of models for cellular response to toxic insult. Beginning with a simple Markov model, successive models increase in biological resolution or refinement as well as mathematical complexity. Our model formulation takes into account broad aspects of the current state of

knowledge concerning the control and regulation of cellular properties in tissue. The design of these models is not to provide a detailed description of the response of specific organs to toxic insult, but rather to begin the process of mathematically describing recent significant advances in knowledge that have been made in cell biology, hormonal regulation, and hormetic control mechanisms that regulate cell response to toxicants. Some modifications of the model formulations may be needed to apply them to specific organs; for one thing the complex geometry of organ architecture is not represented. To have included complex, three-dimensional relationships between different cell types would have increased the mathematical complexity considerably. But even in its present rudimentary state the model is useful for stimulating discussion and suggesting experimentation, with the goal of furthering the understanding of cellular response to toxic substances including the effects of tissue-mediated responses (e.g. mitogenic stimulation). The current formulation of the model, or extensions thereof, is suitable for experimental evaluation in cell culture if the experimental conditions are properly chosen and chemical-specific details are added.

We provide the following abbreviated biological background. The tissue making up organs almost always involves a complex geometrical juxtaposition of several cell types having specific and often overlapping functions. This tissue architecture may be maintained by the presence of a nonliving matrix of proteins collectively known as a *basement membrane*. Interactions between this membrane and the cells are known to be critical to its stability as a mature, functioning entity. The whole of the tissue is permeated by branching blood vessels, each generation of which is successively smaller; these serve to provide a constant, stable milieu in which the cells exist. Nutrients, control signals in the form of specific biomolecules, and xenobiotic chemicals are brought to the immediate

surroundings of the cell by the vasculature in the tissue and cellular metabolic products; the products of energy metabolism and cellularly derived control biomolecules are removed from the cellular microenvironment by the same means.

The state of the cell at any time is a reflection of its age, the summation of the control chemicals reaching and leaving the cell, the effects of xenobiotic chemicals present, if any, and the state of the cells surrounding it. Cellular contact with the surrounding cells and basement membrane act to supply chemical signals that modulate its activity. A given cell may be (i) nearly quiescent, (ii) active biochemically, i.e., producing metabolites of absorbed materials for its own use or for export, (iii) in a state of stress due to shortage or excess of biochemical molecules, (iv) in a process of programmed cell death (apoptosis), (v) in the process of dying from chemical insult (necrosis), or (vi) dividing to form progenitor cells in response to a need to replace cells already lost; these conditions need not be mutually exclusive at any point in time. Some specific cells may alter or completely change their observable characteristics in response to chemical signals. The most well-known of these cells are the pluripotent stem cells of the hematopoietic system.

The models to be presented here represent some, but by no means all, of the features mentioned. They provide a basis for proceeding further.

2. Model Structure

In the following sections of this paper we present a sequence of mathematical models that represent interactions between the cells in an organ's tissue and a toxic chemical or agent (hereafter called *toxin*) originating externally and entering the organ and coming into contact with the cells, possibly in modified form. The initial models postulate a mature organ with capacity to contain, at most, a fixed

number of cells. Broadly speaking, the organ's cells attempt to remove (e.g. metabolize or bind) the toxin, but are, to a dose-dependent degree, also affected by the toxin concentration. We distinguish between cells that are in one of two stages of the cell cycle: these we call, oversimplifying the true cell cycle, *functional*, and *dividing* (S-phase and mitotic). Functional cells are assumed to be capable of removing toxin, but are also susceptible to premature death because of the toxin's action. Dividing cells are in the process of DNA replication and actual mitosis, here splitting into two daughter cells; while in this stage the cells are assumed incapable of removing toxin. However, toxin presence is assumed to affect the cell cycle, e.g. by shortening the functional, and lengthening the dividing, stages. The model proposed emphasizes inter-cellular signaling: when a cell dies, i.e. ceases to function, it effectively requests one other functioning cell to divide to produce its replacement. It is biologically plausible that signaling occurs only to neighboring cells, but the present model, being without spatial characteristics, does not literally recognize this restriction. Also, signals are assumed to be specific: one signal is emitted per cell death, so eventually one replacement occurs. Alternative formulations are sometimes plausible and are easily studied.

3. Single-Stage Markov Model

We now present several alternative simple Markov models for cell-toxin interaction, emphasizing the representation of signaling. Although cells can age and exist in different stages of functionality we omit an account of this at first, but return to it later.

Let $D(t)$ represent the number of functional cells in the organ at time t , and $M(t)$ the number of dividing (mitotic) cells. If a functioning cell that dies immediately induces another to begin division (instant signaling) then

$$C_0 = D(t) + 2M(t) \quad (3.1)$$

where C_0 is the maximum organ size, here assumed constant; realistic elaborations are possible. Note that organ size is, strictly speaking, $D(t) + M(t)$, which is the number of cells present at time t ; C_0 acknowledges that space for all cells must leave room for each cell currently in mitosis to eventually become two differentiated cells. We describe $\{D(t), M(t)\}$ as a birth and death process in continuous time, with $D(t) \in (0, 1, 2, \dots, C_0)$. Typically C_0 is large, i.e. of order $10^{10}-10^{12}$ for mature humans, and this will be exploited to carry out an asymptotic analysis.

Let $T(t)$ denote the concentration of toxin in the organ at time t . We represent $\{T(t)\}$ as a diffusion process whose drift and diffusion coefficients are both influenced by $D(t)$ so as to represent metabolic or binding action, hence toxin removal, and by the toxin input to the organ, $\tau(t)$, as well as the instantaneous concentration, $T(t)$, itself.

In the following we describe specific models, beginning with the most simple and (potentially) least realistic, but also the most parsimonious. We explicitly include the effect of inter-cellular signaling.

3.1 Cellular Signaling

Suppose a cell dies from some cause: apoptosis or necrosis. The effect of its signaling to other cells for replacement can be captured by postulating that a *ghost* (release of growth factor) of the newly-dead cell now exists, the function of which is to find and interact with a neighboring live cell, inducing that cell to divide. The ghost then disappears after its purpose is served. One can thus represent the delay in mitotic response to cell death by adjusting the ghost's search rate for functional cells.

The following describes a possible Markov generator that includes the signaling (ghost) influence. Let $G(t)$ denote the number of signals present in the organism at time t , and introduce the constraint

$$C_0 = D(t) + G(t) + 2M(t). \quad (3.2)$$

The following then describes the possible transitions allowed to occur in a small time interval, $(t, t + dt)$.

**Signaling Model
(Markov Generator)**

t	$t + dt$	Probability
$D(t), G(t), M(t); \rightarrow$	$D(t) - 1, G(t) + 1, M(t)$	$\lambda^* D(t) dt$
$T(t)$	$T(t) + \delta^* dt + \sigma dW(t)$	
	(cell death, signal creation)	
\rightarrow	$D(t) - 1, G(t) - 1, M(t) + 1$	$\theta^* G(t)(D(t)/C_0) dt$
	$T(t) + \delta^* dt + \sigma dW(t)$	
	(cell signaled to divide, signal disappears)	
\rightarrow	$D(t) + 2, G(t), M(t) - 1$	$\mu^* M(t) dt$
	$T(t) + \delta^* dt + \sigma dW(t)$	
	(two newly-divided cells emerge)	

All rates of cell transition, namely λ^* , θ^* , and μ^* are presumed to be influenced by current toxin concentration, $T(t)$. In turn, the latter is influenced by the metabolic or binding capability of the cells to remove toxin; the net mean increase of toxin in the short run is $\delta^*(T(t), D(t), \pi(t))$. Our subsequent analysis does not, in principle, depend upon specific functional forms for any of the above parameters; adequate smoothness and differentiability is assumed where necessary. When specific functions are required we shall use these:

$$\lambda^*(T(t)) = \lambda_0 e^{\lambda^* T(t)} \quad (3.4,a)$$

$$\mu^*(T(t)) = \mu_0 e^{-\mu^* T(t)} \quad (3.4,b)$$

$$\theta^*(T(t)) = \theta_0 e^{-\theta^* T(t)}; \quad \lambda^*, \theta^*, \mu^* > 0. \quad (3.4,c)$$

Note that it has been assumed that increased toxin level increases cell death rate, decreases the rate of completing mitosis, and slows the rate at which ambient signals reach their destinations. The net effect is to reduce the number of functional cells. All of the above dependencies are hypothetical and illustrative only. In particular

$$\delta^*(T(t), D(t), \tau^*(t)) = \left(\tau^*(t) - v^* \frac{T(t)(D(t)/C_0)}{1 + \kappa^* T(t)} \right) \quad (3.5)$$

where in the latter $\tau^*(t)$ represents toxin input rate to the organ, and the remaining term $v^*(T(t)D(t)/C_0)/(1 + \kappa^* T(t))$ represents the toxin concentration rate-limited (Michaelis-Menton) reduction by functional cells.

It has been observed that the realistic effect of toxin presence on λ^* , μ^* , θ^* , etc. at time t may involve the entire past history of such exposure, e.g. $\lambda^*(t) = \lambda^*(T(x), a \leq x \leq t)$; the explicit function may be illustrated by the form $\lambda^*(t) = \exp \left[\int_0^t \lambda^*(T(x) dx) \right]$. A further model enhancement would recognize that toxin presence in the organ may result in cells that complete completing mitosis giving rise to damaged cells, e.g. possessing DNA adducts. These cells now become susceptible to repair.

3.2 Differential Equations For Deterministic Approximations

Let C_0 denote the number of spaces/holes that cells may occupy. Then

$$C_0 = D(t) + G(t) + 2M(t) \quad (3.6)$$

since the cells in division potentially require two holes. Assume that if $C_0 \gg 1$ then

$$D(t)/C_0 \rightarrow \alpha(t), G(t)/C_0 \rightarrow \gamma(t), T(t)/C_0 \rightarrow \beta(t) \quad (3.7)$$

in probability as $C_0 \rightarrow \infty$ where the latter functions are $O(1)$. The following differential equations result from direct manipulation of (3.3):

$$\frac{d\alpha(t)}{dt} = -\lambda\alpha(t) - \theta\alpha(t)\gamma(t) + 2\mu(1 - \alpha(t) - \gamma(t))\frac{1}{2}; \quad (3.8)$$

the last term occurs by virtue of (3.6). Also,

$$\frac{d\gamma(t)}{dt} = \lambda\alpha(t) - \theta\alpha(t)\gamma(t) \quad (3.9)$$

$$\frac{d\beta}{dt} = \delta = \tau - \frac{v\alpha\beta}{1 + \kappa\beta} \quad (3.10)$$

We re-emphasize that all parameters are implicit functions of (current) toxin level, $T(t)$, and possibly also absolute time, t , which can be viewed as the age of a mature organ, rather than an individual cell. This latter step is not taken in this paper.

Note that in (3.8), (3.9), (3.10) the original parameters of (3.3) must be scaled: $\lambda^*(T(t))$ is replaced by $\lambda(\beta(t))$, $\theta^*(T(t))$ by $\theta(\beta(t))$, $\mu^*(T(t))$ by $\mu(\beta(t))$, $v^*(T(t))$ by $v(\beta(t))$, τ^* by τC_0 , and κ^* by κC_0 .

In Section 6 such scaling is exploited more extensively to deduce stochastic behavior of system state values.

3.3 Solution Without Toxin Input

Suppose no toxin input exists, so $T(t) = 0, \forall t \geq 0$. Then a steady-state solution to (3.8) and (3.9) may occur: set derivatives = 0 and solve the resulting equations to find

$$\gamma = \frac{\lambda}{\theta}, \quad \text{provided } 0 \leq \frac{\lambda}{\theta} < 1 \quad (3.11)$$

$$\alpha = \frac{\mu(1 - \lambda/\theta)}{2\lambda + \mu}. \quad (3.12)$$

The latter implies (i) that the larger the signal activity rate, θ , the smaller the ambient signal population, and the more quickly does signaling induce another cell to begin division; furthermore (ii) if $\theta \gg \lambda$, the death rate, signifying highly efficient signaling, then $\alpha \rightarrow \mu/(2\lambda + \mu)$, which is equivalent to thinking of cells as behaving in pairs: once division completes the two daughters effectively compete to die (rate 2λ).

Although the equations (3.8) and (3.9) are non-linear and apparently cannot be solved explicitly one can obtain an approximate time-dependent solution as follows:

(a) assume $\chi(t)$ quickly reaches steady state, so

$$\gamma(t) \equiv \lambda/\theta; \quad 0 < t \quad (3.13)$$

This is a standard and useful approximation technique often invoked in biochemical kinetics problems that is variously abbreviated the *quasi-steady-state assumption* (QSSA) or the *pseudo-steady-state hypothesis* (PSSH); see Segel and Slemrod (1989) for a careful exposition of its rationale. The QSSA approach gives rise to the classical Michaelis-Menton used widely in pharmaceutical kinetics and compartment models; it already appears implicitly in our (3.5) and (3.10).

(b) introduce (3.13) into (3.8) to find the approximation $\tilde{\alpha}(t)$:

$$d\tilde{\alpha}(t)/dt = -(2\lambda + \mu)\alpha + \mu(1 - \lambda/\theta), \quad (3.14)$$

the solution of which is

$$\begin{aligned} \tilde{\alpha}(t) &= \tilde{\alpha}(0)e^{-(2\lambda+\mu)t} + [\mu(1-\lambda/\theta)/(2\lambda+\mu)] \left[1 - e^{-(2\lambda+\mu)t} \right] \\ &\rightarrow \mu(1-\lambda/\theta)/(2\lambda+\mu) \text{ as } t \rightarrow \infty, \end{aligned} \quad (3.15)$$

as in (3.12).

3.4 Dose-Response for Small Steady Toxin Exposure

Of interest in risk analysis is the behavior of the dose response curve for small values of (toxic) dose. We approximate this by finding expressions for $\frac{d\alpha(\tau)}{d\tau} \Big|_{\tau=0}$, $\frac{d\gamma(\tau)}{d\tau} \Big|_{\tau=0}$, and $\frac{d\beta(\tau)}{d\tau} \Big|_{\tau=0}$.

Suppose $\tau(t) = \tau = \tau \sim 0$, a constant, and that exposure has proceeded for some time so that a steady-state condition has been reached; this is modeled by letting $\frac{d\alpha(t)}{dt} = \frac{d\gamma(t)}{dt} = \frac{d\beta}{dt} = 0$ in (3.8) – (3.10). To study the dose-response for $\tau \approx 0$, differentiate (3.10) with respect to τ using the function δ in (3.5) (drop subscript “o” for convenience)

$$0 = 1 - \frac{dv}{d\beta} \frac{d\beta}{d\tau} \frac{\alpha\beta}{1+\kappa\beta} - \frac{v d\alpha}{d\tau} \frac{\beta}{1+\kappa\beta} - \frac{v\alpha}{1+\kappa\beta} \frac{d\beta}{d\tau} + \frac{v\alpha\beta}{(1+\kappa\beta)^2} \frac{d\beta}{d\tau}. \quad (3.16)$$

Since $\beta = 0$ for $\tau = 0$, equation (3.6) yields for the (scaled) rate of toxin concentration at low exposure

$$\beta_\tau \equiv \frac{\partial \beta}{\partial \tau} \Big|_{\tau=0} = \frac{1}{v\alpha} \Big|_{\tau=0} = \frac{1}{v} \frac{2\lambda + \mu}{\mu(1 - \frac{\lambda}{\theta})} > 0 \quad \text{if } \frac{\lambda}{\theta} < 1. \quad (3.17)$$

This shows explicitly how low-dose toxin level in the organ tissue increases as a function of cell-cycle parameters λ and μ , signaling efficiency, θ , and toxin

metabolic or binding rate v . All of these parameters are evaluated at very low (zero) toxin levels.

Next consider steady-state rate of change of scaled functional cell fraction, $\frac{\partial \alpha}{\partial \tau} = \alpha_\tau = \alpha_\beta \cdot \beta_\tau$; this is the slope of a dose-(functioning cell count) response curve at very low levels of toxin. If (3.12) is differentiated and some rearrangements made, and if $\gamma = \lambda/\theta$ as in (3.11), then

$$\alpha_\beta = [-\lambda_\beta \mu (2 + \lambda/\mu + 2(1-\gamma)) + \mu_\beta (2\lambda(1-\gamma)) + \theta_\beta (2\lambda + \mu)(\mu/\theta)\gamma] \frac{1}{(2\lambda + \mu)^2}. \quad (3.18)$$

Since $\beta_\tau > 0$, the sign of α_τ is determined by the above linear combination of the derivatives of the cell cycle and signaling parameters λ , μ , and θ at τ , and hence β , equal to zero. Note that if the illustrative relations (3.4) are invoked as given, then $\alpha_\beta < 0$, hence $\alpha_\tau < 0$ so a small increase in toxin induces a decline in the number of functioning cells; this is intuitively acceptable. However, a change in sign of any of the parameters' derivatives is capable of reversing the sign of the slope of the dose response curve, thus representing a tendency for hormesis at low dose.

4. Models with Cell Aging

In this section we generalize the models of the last section to allow cells to age, and hence exist in possibly different stages of functional effectiveness.

We restrict attention to just two age stages, which we term *new* (n) and *old* (o), distinguishing them by subscript. An arbitrary number of such age states can be introduced, but at the cost of increasing the number of parameters.

Let $D_n(t)$ (respectively $D_o(t)$) represent the number of new (respectively old) cells at time t . $M(t)$ represents the number of dividing (mitotic) cells at time t and $G(t)$ the number of ambient signals or ghosts as before. The spatial constraint is

$$C_0 = D_n(t) + D_o(t) + G(t) + 2M(t). \quad (4.1)$$

Here are the transition probabilities assumed:

Signaling Model with Age Dependence (Markov Generator)

t	$t + dt$	<i>Probability</i>
$D_n(t), D_o(t),$	$\rightarrow D_n(t) - 1, D_o(t), G(t) + 1, M(t)$	$\lambda_n^* D_n(t) dt$
$G(t), M(t);$	$T(t) + \delta^* dt + \alpha dW(t)$	
$T(t)$	(new cell death, signal creation)	
\rightarrow	$D_n(t), D_o(t) - 1, G(t) + 1, M(t)$	$\lambda_o^* D_o(t) dt$
	$T(t) + \delta^* dt + \alpha dW(t)$	
	(old cell death, signal creation)	1
\rightarrow	$D_n(t) - 1, D_o(t), G(t) - 1, M(t) + 1$	$\theta_n^* G(t)(D_n(t)/C_0) dt$
	$T(t) + \delta^* dt + \alpha dW(t)$	
	(new cell signaled to divide, signal disappears)	
\rightarrow	$D_n(t), D_o(t) - 1, G(t) - 1, M(t) + 1$	$\theta_o^* G(t)(D_o(t)/C_0) dt \quad (4.2)$
	$T(t) + \delta^* dt + \alpha dW(t)$	
	(old cell signaled to divide, signal disappears)	
\rightarrow	$D_n(t) + 2, D_o(t), G(t), M(t) - 1$	$\mu^* M(t) dt$
	$T(t) + \delta^* dt + \alpha dW(t)$	
	(two newly-divided new cells emerge)	
\rightarrow	$D_n(t) - 1, D_o(t) + 1, G(t), M(t)$	$\phi^* D_n(t) dt$
	$T(t) + \delta^* dt + \alpha dW(t)$	
	(a new cell becomes an old cell)	

Once again all rates of cell transition, namely λ_n^* , λ_o^* , θ_n^* , θ_o^* , μ^* are presumed to be influenced by current toxin concentration. In turn, the latter is influenced by

the metabolic capability of the cells to remove toxin, $\delta^*(T(t), D_n(t), D_o(t), \tau(t))$. When specific functions are required, we shall use for illustration

$$\delta^*(T(t), D_n(t), D_o(t), \tau(t)) = \tau^*(t) - v_n^* \frac{T(t)D_n(t)/C_0}{1 + \kappa_n^* T(t)} - v_o^* \frac{T(t)D_o(t)/C_0}{1 + \kappa_o^* T(t)} \quad (4.3a)$$

$$\lambda_n^*(T(t)) = \lambda_{n,0} \left(p \left(e^{\lambda_{n,1}^* T(t)} - 1 \right)^+ + q \right) \quad p + q = 1, \quad p \geq 0 \quad (4.3b)$$

$$\lambda_o^*(T(t)) = \lambda_{o,0} e^{\lambda_{o,1}^* T(t)} \quad (4.3c)$$

$$\phi^*(T(t)) = \phi_0 e^{\phi_1^* T(t)} \quad (4.3d)$$

$$\mu^*(T(t)) = \mu_0 e^{-\mu_1^* T(t)} \quad (4.3e)$$

The form of (4.3b) permits the adjustment of organ toxin concentration-caused cell death rate to be flexibly adjusted; if $\lambda_{n,1}^* < 0$ the inner bracket must have its sign reversed: $(\)^+$. As before, the above forms are hypothetical.

4.1 Differential Equations for Deterministic Approximations

Let C_0 denote the number of spaces/holes that cells may occupy. Then

$$C_0 = D_n(t) + D_o(t) + G(t) + 2M(t).$$

Assume that

$$\frac{D_n(t)}{C_0} \rightarrow \alpha_n(t), \quad \frac{D_o(t)}{C_0} \rightarrow \alpha_o(t), \quad \frac{G(t)}{C_0} \rightarrow \gamma(t), \quad \frac{T(t)}{C_0} \rightarrow \beta(t) \quad (4.4)$$

in probability as $C_0 \rightarrow \infty$. The following differential equations result from direct manipulation of (4.2) with scaled rate functions

$$\frac{d\alpha_n(t)}{dt} = -[\lambda_n + \phi]\alpha_n(t) - \theta_n \alpha_n(t)\gamma(t) + \mu[1 - \alpha_n(t) - \alpha_o(t) - \gamma(t)] \quad (4.5)$$

$$\frac{d\alpha_o(t)}{dt} = -\lambda_o \alpha_o(t) + \phi \alpha_n(t) - \theta_o \alpha_o(t)\gamma(t) \quad (4.6)$$

$$\frac{d\gamma(t)}{dt} = -[\theta_n \alpha_n(t) + \theta_o \alpha_o(t)]\gamma(t) + \lambda_n \alpha_n(t) + \lambda_o \alpha_o(t) \quad (4.7)$$

$$\frac{d\beta(t)}{dt} = \tau(t) - v_n \alpha_n(t) \frac{\beta(t)}{1 + \kappa_n \beta(t)} - v_o \alpha_o(t) \frac{\beta(t)}{1 + \kappa_o \beta(t)} \quad (4.8)$$

These equations can be solved numerically, but not in closed parametric form.

4.2 Solution Without Toxin Input

Suppose no toxin input exists so $T(t) = 0$, $t \geq 0$. Then a steady-state solution to (4.5) – (4.7) may occur; set derivatives equal to 0 obtaining the following equations.

$$0 = -(\lambda_n + \phi)\alpha_n - \theta_n \alpha_n \gamma + \mu[1 - \alpha_n - \alpha_o - \gamma] \quad (4.9)$$

$$0 = -\lambda_o \alpha_o + \phi \alpha_n - \theta_o \alpha_o \gamma \quad (4.10)$$

$$0 = [-\theta_n \alpha_n - \theta_o \alpha_o] \gamma + \lambda_n \alpha_n + \lambda_o \alpha_o. \quad (4.11)$$

Equation (4.10) yields

$$\alpha_n = (1/\phi)[\lambda_o + \theta_o \gamma] \alpha_o. \quad (4.12)$$

Substituting (4.12) into equation (4.11) yields

$$0 = [-\theta_n(1/\phi)[\lambda_o + \theta_o \gamma] - \theta_o] \gamma + (\lambda_n[(1/\phi)[\lambda_o + \theta_o \gamma]] + \lambda_o) \quad (4.13)$$

after dividing through by α_o .

Rewriting (4.13) we obtain

$$0 = (-\theta_n \theta_o / \phi) \gamma^2 - \gamma [\theta_n(1/\phi) \lambda_o + \theta_o - \lambda_n(1/\phi) \theta_o] + [(\lambda_n/\phi) + 1] \lambda_o. \quad (4.14)$$

Thus γ satisfies a quadratic equation. Let γ_o be the positive solution to the equation. If $\lambda_n(0) = 0$, as in (4.3b)

$$\gamma_o = \frac{-\left[\frac{\lambda_o}{\theta_o} + \frac{\phi}{\theta_n}\right] + \sqrt{\left(\frac{\lambda_o}{\theta_o} + \frac{\phi}{\theta_n}\right)^2 + 4 \frac{\lambda_o}{\theta_o} \frac{\phi}{\theta_n}}}{2}. \quad (4.15)$$

If $\gamma_0 < 1$, then $\gamma = \gamma_0$. If $\gamma_0 \geq 1$ then ambient signals and not functional cells dominate the organ, which in reality would be long defunct even without toxin.

If $\frac{\theta_n}{\phi} < 1$ and

$$\frac{\lambda_o}{\theta_o} < \left[1 + \frac{\theta_n}{\phi} \right] / \left[1 - \frac{\theta_n}{\phi} \right] \quad (4.16)$$

then $\gamma_0 < 1$.

To find α_o equation (4.9) is used. Put $c_1 = (1/\phi)[\lambda_o + \theta_o\gamma]$. Then

$$\mu[1 - \gamma_o] = \{(\lambda_n + \phi)c_1 + \theta_n\gamma c_1 + \mu[c_1 + 1]\}\alpha_o = \{2\lambda_o + \mu[c_1 + 1] + 2\lambda_n c_1\}\alpha_o.$$

Thus,

$$\alpha_o = \mu[1 - \gamma_o] / \{2\lambda_o + \mu[1 + c_1] + 2\lambda_n c_1\} \quad (4.17)$$

and

$$\alpha_n = c_1 \alpha_o. \quad (4.18)$$

4.3 Dose-Response For Small Steady Toxin Input

As mentioned earlier, the behavior of an organ-level dose-response curve for small values of toxic dose is of interest in risk analysis. In this section we indicate that the derivative of various cellular-level responses with respect to τ may be evaluated at $\tau = 0$.

If $\tau = 0$ then $\beta = 0$. Set the left-hand side of (4.8) equal to zero, then differentiate the right side with respect to τ . After setting $\beta = 0$, this yields

$$0 = 1 - [v_n \alpha_n + v_o \alpha_o] \frac{d\beta}{d\tau}. \quad (4.19)$$

Thus

$$\left. \frac{d\beta}{d\tau} \right|_{\tau=0} = \frac{1}{v_n \alpha_n + v_o \alpha_o} > 0, \quad (4.20)$$

as is physically plausible.

It may be shown by further differentiation that under the specific parameterization of (3.4), or suitable alternatives, all derivatives of long-run cellular-level response can be explicitly evaluated at $\tau = 0$, as was shown earlier. The complex details are omitted here.

5. A Model with Cell Aging and Organ Tissue Growth

In this section we generalize the previous model to allow cells to spontaneously enter mitosis and multiply, thus effectively increasing the organ size, where the latter is defined as the number of cells in existence, in either state. In the previous section cell replication only occurred to replace other cells that previously have died, possibly as a result of toxin action.

Consider the region to be occupied by cells to consist of spaces/holes that may be filled by cells. Let C_0 be the maximum number of such holes, in this case the number in an essentially mature organ. Initially not all such holes are *active*, for the organ is immature and hence growing. Hence let $C(t)$ be the number of active holes at time t , while $C_0 - C(t)$ are currently quiescent or inactive. Now, generalizing the previous setup, let the current number of active holes/spaces be

$$C(t) = D_n(t) + D_o(t) + G(t) + 2M(t) \quad (5.1)$$

and augment the previous model to allow $C(t)$ to gradually grow, although remaining bounded by C_0 .

5.1 Organ Growth Stimulated by Signaling

In this model the presence of inactive holes/spaces effectively encourages the active cells, $D_n(t)$ and $D_o(t)$, to increase the number of signals and hence the number of active spaces capable of accomodating cells. We are implicitly modeling the competing effects of positive and negative, or inhibitory, growth factors.

The model is specified by the following transitions.

Signaling-Driven Organ Growth (Markov Generator)

t	$t + dt$	<i>Probability</i>
$D_n(t), D_o(t), G(t), M(t), C(t)$ $G(t), M(t),$ $C(t) T(t)$	$D_n(t), D_o(t), G(t) + 1, M(t), C(t) + 1$ $T(t) + \delta dt + \alpha dW(t)$ (a quiescent cell space becomes active, signal creation)	$\left[\xi_n^* D_n(t) + \xi_o^* D_o(t) \right] \left[1 - \frac{C(t)}{C_o} \right] dt$
	$\rightarrow D_n(t) - 1, D_o(t), G(t) + 1, M(t), C(t)$ $T(t) + \delta dt + \alpha dW(t)$ (new cell death, signal creation)	$\lambda_n^* D_n(t) dt$
	$\rightarrow D_n(t), D_o(t) - 1, G(t) + 1, M(t), C(t)$ $T(t) + \delta dt + \alpha dW(t)$ (old cell death, signal creation)	$\lambda_o^* D_o(t) dt$
	$\rightarrow D_n(t) + 2, D_o(t), G(t) + 1, M(t) - 1, C(t)$ $T(t) + \delta dt + \alpha dW(t)$ (two newly-divided cells emerge)	$\mu^* M(t) dt$
	$\rightarrow D_n(t) - 1, D_o(t) + 1, G(t), M(t), C(t)$ $T(t) + \delta dt + \alpha dW(t)$ (new cell becomes old)	$\phi^* D_n(t) dt$
	$\rightarrow D_n(t) - 1, D_o(t), G(t) - 1, M(t) + 1, C(t)$ $T(t) + \delta dt + \alpha dW(t)$ (new cell signaled to divide, signal vanishes)	$\theta_n^* G(t) \frac{D_n(t)}{C(t)} dt$
	$\rightarrow D_n(t), D_o(t) - 1, G(t) - 1, M(t) + 1, C(t)$ $T(t) + \delta dt + \alpha dW(t)$ (old cell signaled to divide, signal vanishes)	$\theta_o^* G(t) \frac{D_o(t)}{C(t)} dt$

Once again, all rates of cell transition, namely, λ_n^* , λ_o^* , θ^* , ϕ^* , μ^* , ξ_n^* , ξ_o^* are presumed to be influenced by current toxin concentration. When specific functions are required we shall use

$$\begin{aligned}\xi_n^*(T(t)) &= \xi_{n,0} e^{-\xi_{n,1}^* T(t)} \\ \xi_o^*(T(t)) &= \xi_{o,0} e^{-\xi_{o,1}^* T(t)}\end{aligned}\quad (5.3)$$

in addition to those of (4.3 a-e).

Once again assume that as $C_0 \rightarrow \infty$

$$\frac{D_n(t)}{C_0} \rightarrow \alpha_n(t), \quad \frac{D_o(t)}{C_0} \rightarrow \alpha_o(t), \quad \frac{G(t)}{C_0} \rightarrow \gamma(t), \quad \frac{C(t)}{C_0} \rightarrow \eta(t), \quad \frac{T(t)}{C_0} \rightarrow \beta(t) \quad (5.4)$$

in probability.

The following differential equations result from direct manipulation of (5.2).

$$\frac{d\alpha_n(t)}{dt} = -[\lambda_n + \phi]\alpha_n(t) - \theta_n \frac{\alpha_n(t)}{\eta(t)} \gamma(t) + \mu(\eta(t) - \alpha_n(t) - \alpha_o(t) - \gamma(t)) \quad (5.5)$$

$$\frac{d\alpha_o(t)}{dt} = -\lambda_o \alpha_o(t) - \theta_o \frac{\alpha_o(t)}{\eta(t)} \gamma(t) + \phi \alpha_n(t) \quad (5.6)$$

$$\frac{d\gamma(t)}{dt} = -\frac{[\theta_n \alpha_n(t) + \theta_o \alpha_o(t)]}{\eta(t)} \gamma(t) + \lambda_n \alpha_n(t) + \lambda_o \alpha_o(t) + [\xi_o \alpha_o(t) + \xi_n \alpha_n(t)][1 - \eta(t)] \quad (5.7)$$

$$\frac{d\eta(t)}{dt} = [\xi_n \alpha_n(t) + \xi_o \alpha_o(t)][1 - \eta(t)] \quad (5.8)$$

$$\frac{d\beta(t)}{dt} = \delta \quad (5.9)$$

6. Stochastic Differential Equation Models

The size of the state space makes the time-dependent behavior of the continuous-time Markov chain models of Sections 3 – 5 difficult to study. One

approach to studying the behavior is by Monte Carlo simulation; cf. Bois, et al. (1992). Another approach, adopted here, is to approximate the continuous-time Markov chain model by a diffusion process. Note that the continuous-time Markov chain model has absorbing states; for example, any state with $D_n(t) + D_o(t) = 0$ is absorbing. Of course no self-respecting organ would ever start life in such a state, and a living organ would presumably die from other unmodeled causes long before such a state is reached, i.e. when $D_n(t) + D_o(t) \leq D$, some lower limit. Barbour (1976) discusses how long and over what ranges the underlying continuous-time Markov chain is approximated by a diffusion process of the type we derive; see also discussion to McNeil and Schach (1973). Stochastic differential equation models can be written for all the models of Sections 3 – 5. We will illustrate our approach by writing down a system of stochastic differential equations for the multivariate process $(D_n(t), D_o(t), G(t), C(t), T(t))$ for Markov generator (5.2). The system of stochastic differential equations is as follows; as explained subsequently, the dW -terms are normally distributed.

$$dD_n(t) = -\lambda_n^*(T(t))D_n(t)dt - \theta_n^*(T(t))G(t)(D_n(t)/C(t))dt - \phi^*(T(t))D_n(t)dt + \mu^*(T(t))(C(t) - D_n(t) - D_o(t) - G(t))dt \quad \left. \begin{array}{l} \text{drift = conditional} \\ \text{expected change} \\ \text{in } (t, t+dt) \end{array} \right\} \quad (6.1)$$

$$-\sqrt{\varepsilon\lambda_n^*(T(t))D_n(t)}dW_{\lambda_n}(t) - \sqrt{\varepsilon\phi^*(T(t))D_n(t)}dW_\phi(t) \quad \left. \begin{array}{l} \text{diffusion = random} \\ \text{change in } (t, t+dt) \end{array} \right\}$$

$$-\sqrt{\varepsilon\theta_n^*(T(t))G(t)(D_n(t)/C(t))}dW_{\theta_n}(t) + \sqrt{\varepsilon 4\mu^*(T(t))(C(t) - D_n(t) - D_o(t) - G(t))\frac{1}{2}}dW_\mu(t)$$

$$\begin{aligned}
dD_o(t) = & -\lambda_o^*(T(t))D_o(t)dt - \theta_o^*(T(t))G(t)(D_o(t)/C(t))dt \\
& + \phi^*(T(t))D_n(t)dt \\
& - \sqrt{\varepsilon \lambda_o^*(T(t))D_o(t)} dW_{\lambda_o}(t) + \sqrt{\varepsilon \phi^*(T(t))D_n(t)} dW_\phi(t) \\
& - \sqrt{\varepsilon \theta_o^*(T(t))G(t)(D_o(t)/C(t))} dW_{\theta_o}(t)
\end{aligned} \left. \right\} \text{(drift)} \quad (6.2)$$

$$\begin{aligned}
dG(t) = & -\theta_n^*(T(t))G(t)(D_n(t)/C(t))dt - \theta_o^*(T(t))G(t)(D_o(t)/C(t))dt \\
& + \lambda_o^*(T(t))D_o(t)dt + \lambda_n^*(T(t))D_n(t)dt \\
& + \xi_n^*(T(t))D_n(t)\left[1 - \frac{C(t)}{C_0}\right] + \xi_o^*(T(t))D_o(t)\left[1 - \frac{C(t)}{C_0}\right] \\
& - \sqrt{\varepsilon \theta_n^*(T(t))G(t)(D_n(t)/C(t))} dW_{\theta_n}(t) \\
& - \sqrt{\varepsilon \theta_o^*(T(t))G(t)(D_o(t)/C(t))} dW_{\theta_o}(t) \\
& + \sqrt{\varepsilon \lambda_o^*(T(t))D_o(t)} dW_{\lambda_o}(t) + \sqrt{\varepsilon \lambda_n^*(T(t))D_n(t)} dW_{\lambda_n}(t) \\
& + \sqrt{\varepsilon \xi_n^*(T(t))D_n(t)\left[1 - \frac{C(t)}{C_0}\right]} dW_{\xi_n}(t) \\
& + \sqrt{\varepsilon \xi_o^*(T(t))D_o(t)\left[1 - \frac{C(t)}{C_0}\right]} dW_{\xi_o}(t)
\end{aligned} \left. \right\} \text{(diffusion)} \quad (6.3)$$

$$\frac{dC(t)}{dt} = \xi_o^*(T(t))D_o(t) \left[1 - \frac{C(t)}{C_0} \right] + \xi_n^*(T(t))D_n(t) \left[1 - \frac{C(t)}{C_0} \right] \left\{ \begin{array}{l} (\text{drift}) \\ \\ + \sqrt{\varepsilon \xi_o^*(T(t))D_o(t) \left[1 - \frac{C(t)}{C_0} \right]} dW_{\xi_o}(t) \\ \\ + \sqrt{\varepsilon \xi_n^*(T(t))D_n(t) \left[1 - \frac{C(t)}{C_0} \right]} dW_{\xi_n}(t) \end{array} \right\} \quad (6.4)$$

$$\frac{dT(t)}{dt} = \delta^* \left(T(t), D_n(t), D_o(t), \tau^*(t) \right) dt \left\{ \begin{array}{l} (\text{drift}) \\ \\ + \sigma_T^*(T(t), D_n(t), D_o(t), \tau(t)) dW_{\sigma_T}(t) \\ \\ + \sqrt{C_0} \sigma_\tau dW_\tau(t) \end{array} \right\} \quad (6.5)$$

where $\{W_{\lambda_n}(t)\}, \{W_{\lambda_o}(t)\}, \{W_{\theta_n}(t)\}, \{W_{\theta_o}(t)\}, \{W_\mu(t)\}, \{W_{\xi_o}(t)\}, \{W_{\xi_n}(t)\}, \{W_{\sigma_T}(t)\}$, and $\{W_\tau(t)\}$ are independent standard Brownian motions; each $dW(t)$ -term is thus Gaussian with mean zero and variance dt .

Setting the constant $\varepsilon = 1$ lets the variances of the change in the number of active cells, ghosts in identified states, and active spaces be equal to the mean respective changes; this represents Poisson variability. Setting the constant $\varepsilon > 1$ implies that the variability is larger than Poisson variability. This additional variability may be the result of inhomogeneities in the organ that are not explicitly modeled; cf. Bass, Robinson and Bracken (1978) for discussion of a distributed liver model with random inhomogeneities. Such *overdispersion* is frequently encountered in practice, and may be accounted for parsimoniously as we do; see McCullagh and Nelder (1983).

We assume that as $C_0 \rightarrow \infty$

$$\frac{D_n(t)}{C_0} \rightarrow \alpha_n(t), \frac{D_o(t)}{C_0} \rightarrow \alpha_o(t), \frac{G(t)}{C_0} \rightarrow \gamma(t), \frac{C(t)}{C_0} \rightarrow \eta(t), \frac{T(t)}{C_0} \rightarrow \beta(t) \quad (6.6)$$

in probability and rewrite $D_n(t)$, $D_o(t)$, $G(t)$, $C(t)$, and $T(t)$ as follows.

$$D_n(t) = C_0 \alpha_n(t) + \sqrt{C_0} X_n(t) \quad (6.7a)$$

$$D_o(t) = C_0 \alpha_o(t) + \sqrt{C_0} X_o(t) \quad (6.7b)$$

$$G(t) = C_0 \gamma(t) + \sqrt{C_0} X_g(t) \quad (6.7c)$$

$$C(t) = C_0 \eta(t) + \sqrt{C_0} X_c(t) \quad (6.7d)$$

$$T(t) = C_0 \beta(t) + \sqrt{C_0} Y(t) \quad (6.7e)$$

$$d\tau^*(t) = C_0 \tau(t) + \sqrt{C_0} \sigma_\tau dW_\tau(t) \quad (6.7f)$$

The transition rates will be scaled and expanded as follows:

$$\lambda_n^*(T(t)) = \lambda_n(T(t)/C_0) = \lambda_n(\beta(t)) + \lambda'_n(\beta(t)) Y(t) / \sqrt{C_0} + O(1) \quad (6.8a)$$

$$\lambda_o^*(T(t)) = \lambda_o(T(t)/C_0) = \lambda_o(\beta(t)) + \lambda'_o(\beta(t)) Y(t) / \sqrt{C_0} + O(1) \quad (6.8b)$$

$$\mu^*(T(t)) = \mu(T(t)/C_0) = \mu(\beta(t)) + \mu'(\beta(t)) Y(t) / \sqrt{C_0} + O(1) \quad (6.8c)$$

$$\theta_n^*(T(t)) = \theta_n(T(t)/C_0) = \theta_n(\beta(t)) + \theta'_n(\beta(t)) Y(t) / \sqrt{C_0} + O(1) \quad (6.8d)$$

$$\theta_o^*(T(t)) = \theta_o(T(t)/C_0) = \theta_o(\beta(t)) + \theta'_o(\beta(t)) Y(t) / \sqrt{C_0} + O(1) \quad (6.8e)$$

$$\xi_n^*(T(t)) = \xi_n(T(t)/C_0) = \xi_n(\beta(t)) + \xi'_n(\beta(t)) Y(t) / \sqrt{C_0} + O(1) \quad (6.8f)$$

$$\xi_o^*(T(t)) = \xi_o(T(t)/C_0) = \xi_o(\beta(t)) + \xi'_o(\beta(t)) Y(t) / \sqrt{C_0} + O(1) \quad (6.8g)$$

$$\begin{aligned}\delta^*(D_n(t), D_o(t), T(t), \tau^*(t)) &= C_0 \delta(D_n(t)/C_0, D_o(t)/C_0, T(t)/C_0, \tau^*(t)/C_0) \\ &= C_0 \delta(\alpha_n(t), \alpha_o(t), \beta(t), \tau(t))\end{aligned}\quad (6.8h)$$

$$+ C_0 \delta'_{\alpha_n} \frac{X_n(t)}{\sqrt{C_0}} + C_0 \delta'_{\alpha_o} \frac{X_o(t)}{\sqrt{C_0}} + C_0 \delta'_\beta \frac{Y(t)}{\sqrt{C_0}} + O(1)$$

$$\begin{aligned}\sigma_T^*(D_n(t), D_o(t), T(t), \tau^*(t)) &= \sqrt{C_0} \sigma_T(D_n(t)/C_0, D_o(t)/C_0, T(t)/C_0, \tau^*(t)/C_0) \\ &= \sqrt{C_0} \sigma_T(\alpha_n(t), \alpha_o(t), \beta(t), \tau(t)) + O(1)\end{aligned}\quad (6.8i)$$

Dividing equations (6.1) – (6.5) by C_0 , scaling and substituting (6.7a) – (6.7f) results in a system of equations involving the deterministic components $\alpha_n(t)$, $\alpha_o(t)$, $\gamma(t)$, $\eta(t)$, and $\beta(t)$ and the corresponding stochastic components $X_n(t)$, $X_o(t)$, $X_g(t)$, $X_c(t)$, and $Y(t)$. See Appendix 1 for details.

6.1 Second Moments

The stochastic differential equations (A.1) – (A.10) of Appendix 1, derived for the stochastic disturbances, can be written in matrix-vector form as

$$dZ(t) = A(t)Z(t) + B(t) dW(t) \quad (6.9)$$

where in the present example Z is a 5×1 column vector, A is a 5×5 matrix, $dW(t)$ is 10×1 column of independent Gaussian noise terms and $B(t)$ is a 5×10 matrix.

It follows from theorem (8.5.5) of Arnold (1974) that

$$\frac{d}{dt} E[Z_i^2(t)] = 2 \sum_j A_{ij}(t) E[Z_i(t) Z_j(t)] + \sum_j (B_{ij}(t))^2 \quad (6.10)$$

and

$$\begin{aligned}
\frac{d}{dt} E[Z_i(t)Z_j(t)] &= \sum_k \mathbf{A}_{ik}(t)E[Z_k(t)Z_j(t)] \\
&\quad + \sum_k \mathbf{A}_{jk}(t)E[Z_k(t)Z_i(t)] \tag{6.11} \\
&\quad + \sum_k \mathbf{B}_{ik}(t)\mathbf{B}_{jk}(t)
\end{aligned}$$

Thus, the approximate distribution of $D_n(t)$, (respectively $D_o(t)$, $G(t)$, $C(t)$, $T(t)$) is normal with mean $C_0\alpha_n(t)$, (respectively $C_0\alpha_o(t)$, $C_0\gamma(t)$, $C_0\eta(t)$, $C_0\beta(t)$) and variance $C_0E[X_n^2(t)]$, (respectively $C_0E[X_o^2(t)]$, $C_0E[X_g^2(t)]$, $C_0E[X_c^2(t)]$, $C_0E[Y^2(t)]$). The approximate covariance of $D_n(t)$ and $D_o(t)$ is $C_0E[X_n(t)X_o(t)]$, etc.

We point out that exactly equivalent results can be obtained by introducing transforms for the state variables, computed by taking conditional mathematical expectations of exponential functions of the Markov generators. This is the route followed by McNeil and Schach (1973); see also Carpenter, Gaver, and Jacobs (1993). The transform approach provides a verification that the asymptotics described here actually lead to limiting Gaussian distributions and to Ornstein-Uhlenbeck processes. See Ethier and Kurtz (1986) for a more rigorous discussion. The approach taken here, while heuristic, is intuitively appealing and reaches the same ultimate conclusion.

6.2 Numerical Examples

In this section we present three numerical examples.

a. Constant Rate of Input of Toxin

We assume chronic dosage of the organ by toxin that is delivered there at constant mean rate but with substantial randomness around that mean.

Figures 1 – 5 depict the time variation of the expected numbers of new cells, of old cells, of total number of active (new plus old) cells, number of signals

(ghosts), and amount of toxin plus/minus two population standard deviations. There is no organ growth. The initial conditions are the limiting moments with no toxin input. The toxin input rate 0.2 units/cell for all time. The new cells without toxin have mean time 100 before transitioning to old. The old cells have mean lifetime of 100 without toxin. The number of cells in the organ is 1.5×10^8 . The organ size is about that of a mouse liver. Since the cells in a mouse liver have a mean lifetime of about 200 days; cf. Bois, et al. (1992), the above is consistent with mouse liver behavior. The MATLAB 4th and 5th order Runge-Kutta-Fehlberg numerical integration method was used to obtain the solutions. The MATLAB plotting algorithms were used to produce the figures.

There is extra Poisson variability: $\varepsilon = 10^4$.

Note that the total mean number of active (new and old) cells decreases as the result of toxin input, as seems intuitively reasonable. The mean number of new cells initially decreases, but eventually increases to a new steady-state value which is larger than the value with no toxin. The mean number of old cells initially increases briefly, but then decreases to a new steady-state value below the value for no toxin. The mean amount of toxin and the mean number of signals (ghosts) both increase to new steady-state values.

The above hypothetical example illustrates how model parametric inputs translate into an account of the dynamics of subpopulations of cells. The details of the transition from one steady-state situation to another are of possible interest in that the organ may be in jeopardy during that transitional period because of, for example, new cell initial downward fluctuation. The effect on organ mortality is, however, not explicitly modeled.

b. A Toxin Pulse or Bolus Dose

Figures 6 – 10 display the mean number of new cells, old cells, active cells (new and old), signals (ghosts) and amount of toxin plus and minus 2 standard deviations for the case in which the rate of toxin input is 0.2 per cell for the first 50 time units and 0 thereafter. Once again there is no organ growth. The initial conditions are steady-state values when there is no toxin input. Note that the number of active cells decreases during toxin input, then increases and slightly overshoots the steady-state value with no toxin before returning to its steady-state value with no toxin.

c. Organ Growth

The model of Section 5.1 and (6.1) – (6.5) is initialized with the steady-state values for the second moments with no input of toxin. However, only 1/2 of the available spaces are active (may contain cells); the steady-state mean of new and old cells and ghosts for no toxin input are multiplied by 1/2 and used as initial values. Figures 11 – 15 display the mean numbers of new, old, and active cells, ghosts, and active spaces plus/minus two standard deviations for the case of no toxin input and parameters $\xi_{n,0} = 0.5$, $\xi_{n,1} = 0.5$, $\xi_{o,0} = 1$, $\xi_{o,1} = 0.5$. The mean number of active spaces is the maximum number by time 20. However, the mean number of old and new cells is still adjusting by time 100. The mean number of old cells initially decreases before increasing. The mean number of new cells initially increases before decreasing.

The present model might be useful for describing the aftermath of a partial hepatectomy: about one-half of a mature liver remains, and grows back with subpopulations of cells responding as shown. Note that, according to the present model in Figure 12, the organ must withstand an early signal to old cells to undergo substantial depletion to enter mitosis and bring forth replacements; the

latter are the new cells depicted in Figure 11. It may be that the sudden depletion of old cells to their low point at about $t = 6$ (Figure 12) actually puts the organ in jeopardy.

Figures 16 – 21 display the mean number of new, old, and active cells, ghosts and active spaces plus/minus two standard deviations for the same parameters as used in Figures 11 – 15 but with two levels of toxin input. The dashed lines correspond to no toxin input. The solid lines correspond to a constant toxin input of 0.2 per cell per unit time. The graphs with positive toxin input have the same general shape as those with no toxin input. The mean number of new cells is initially less for a positive toxin input than for no toxin input. However, by time 100 the mean number of new cells with positive toxin input is becoming larger than that for no toxin input.

The effect of positive toxin input on the mean number of old cells is that it is initially larger than that for no toxin but by time 100 the mean number of old cells with positive toxin is less than that for no toxin. The effect of positive toxin input on the mean number of active (old and new) cells is to decrease it and delay its approach to a steady state value. The mean number of ghosts is larger with positive toxin input. Positive toxin input delays the overall growth in the number of active spaces.

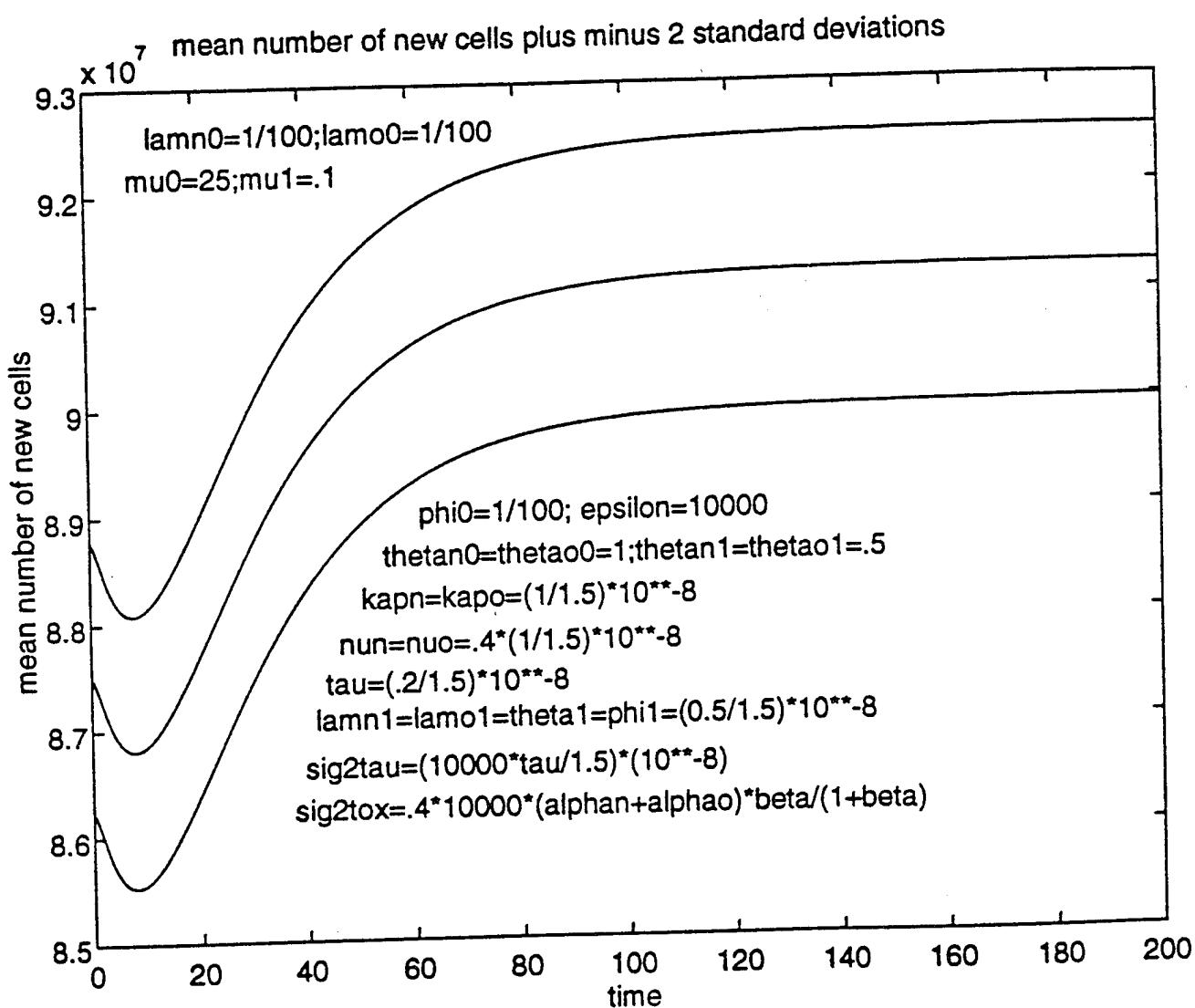


Figure 1

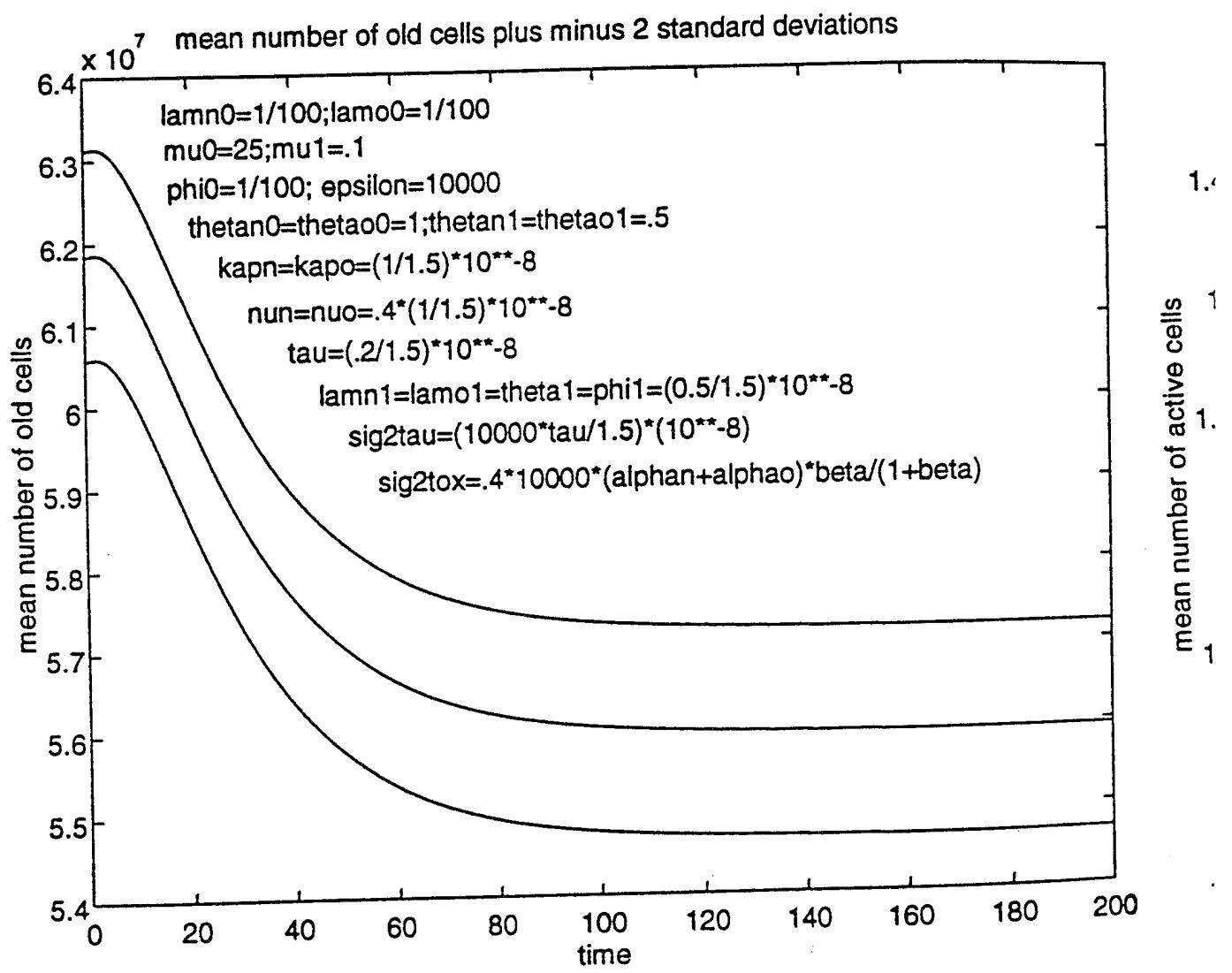


Figure 2

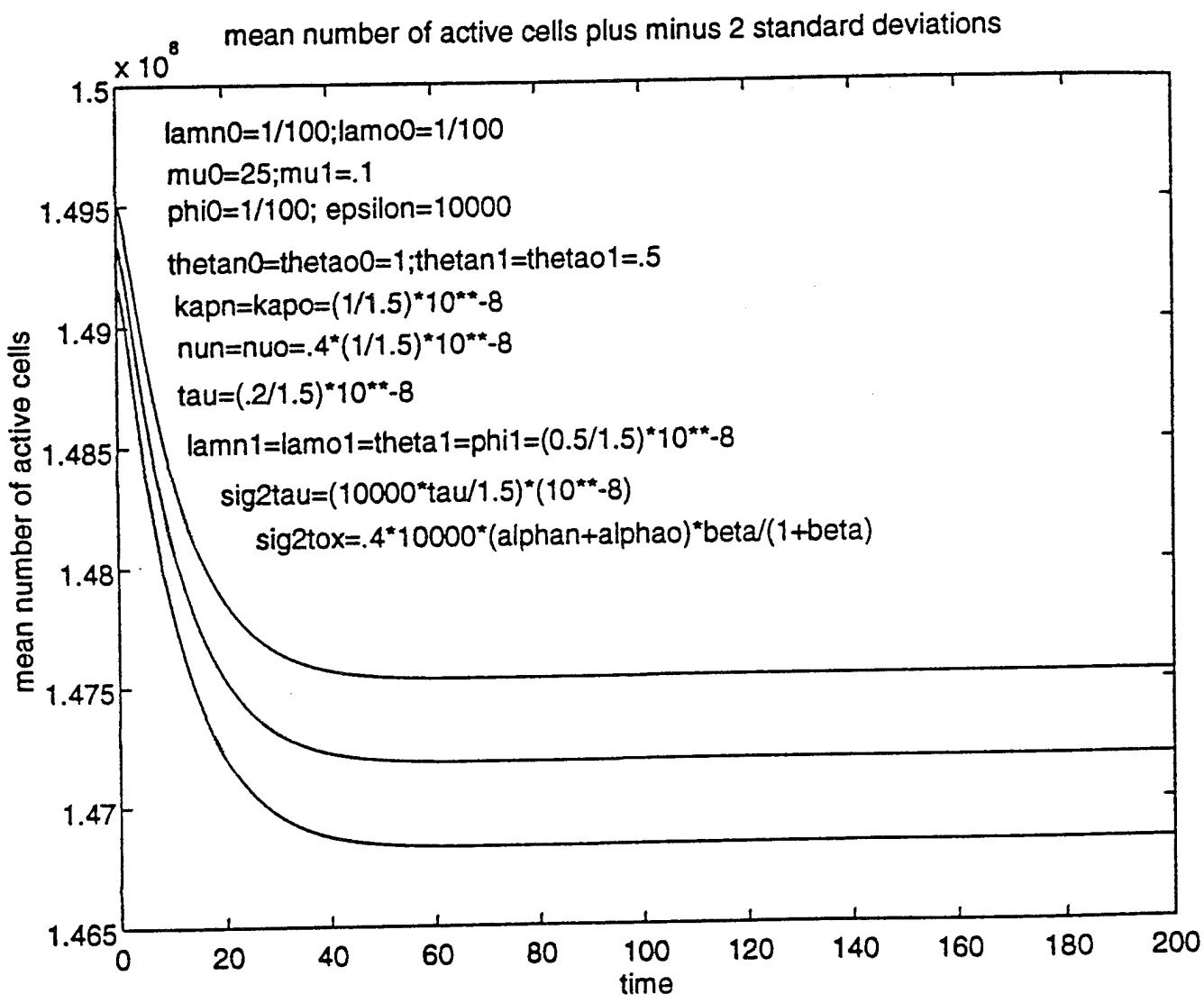


Figure 3

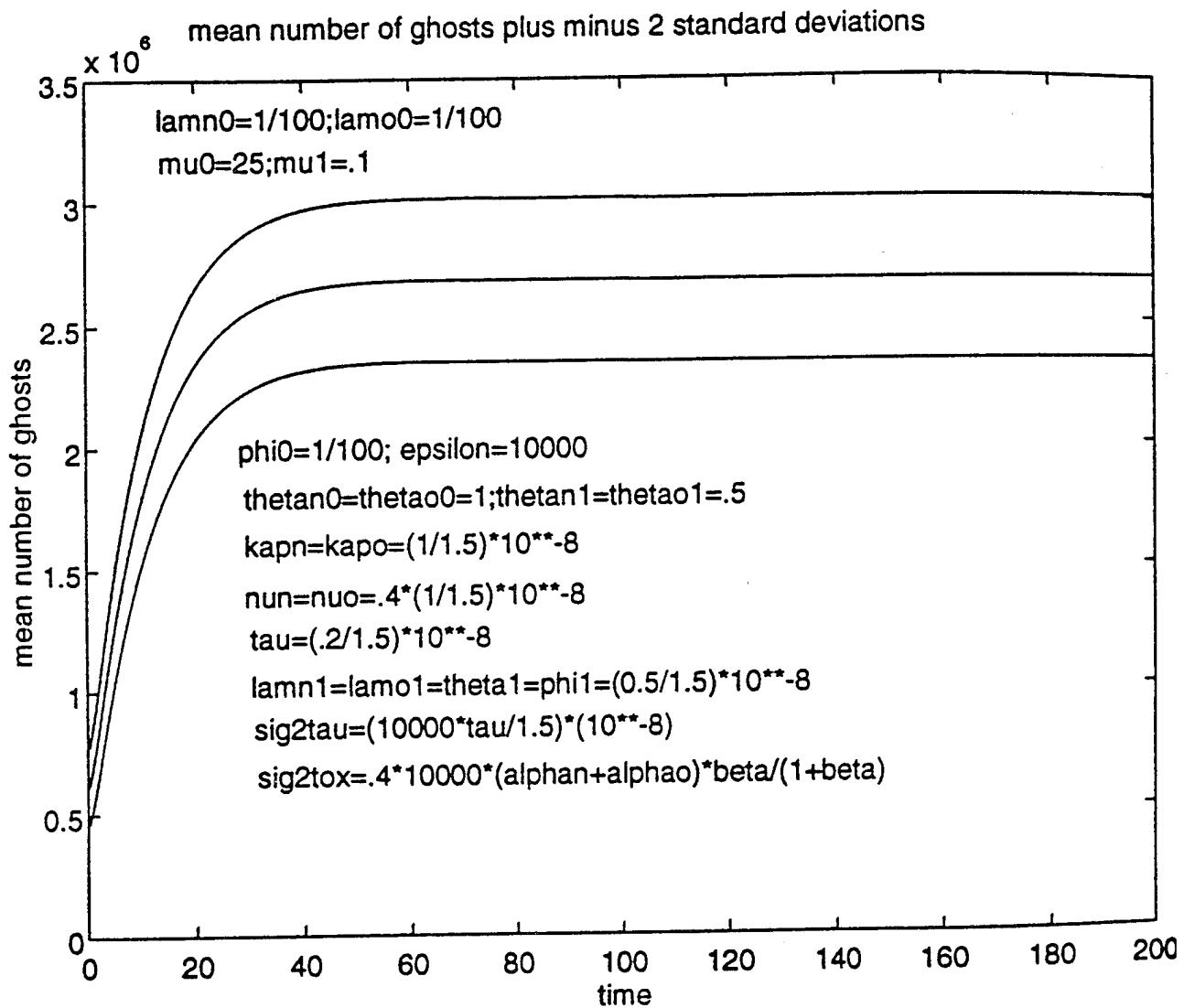


Figure 4

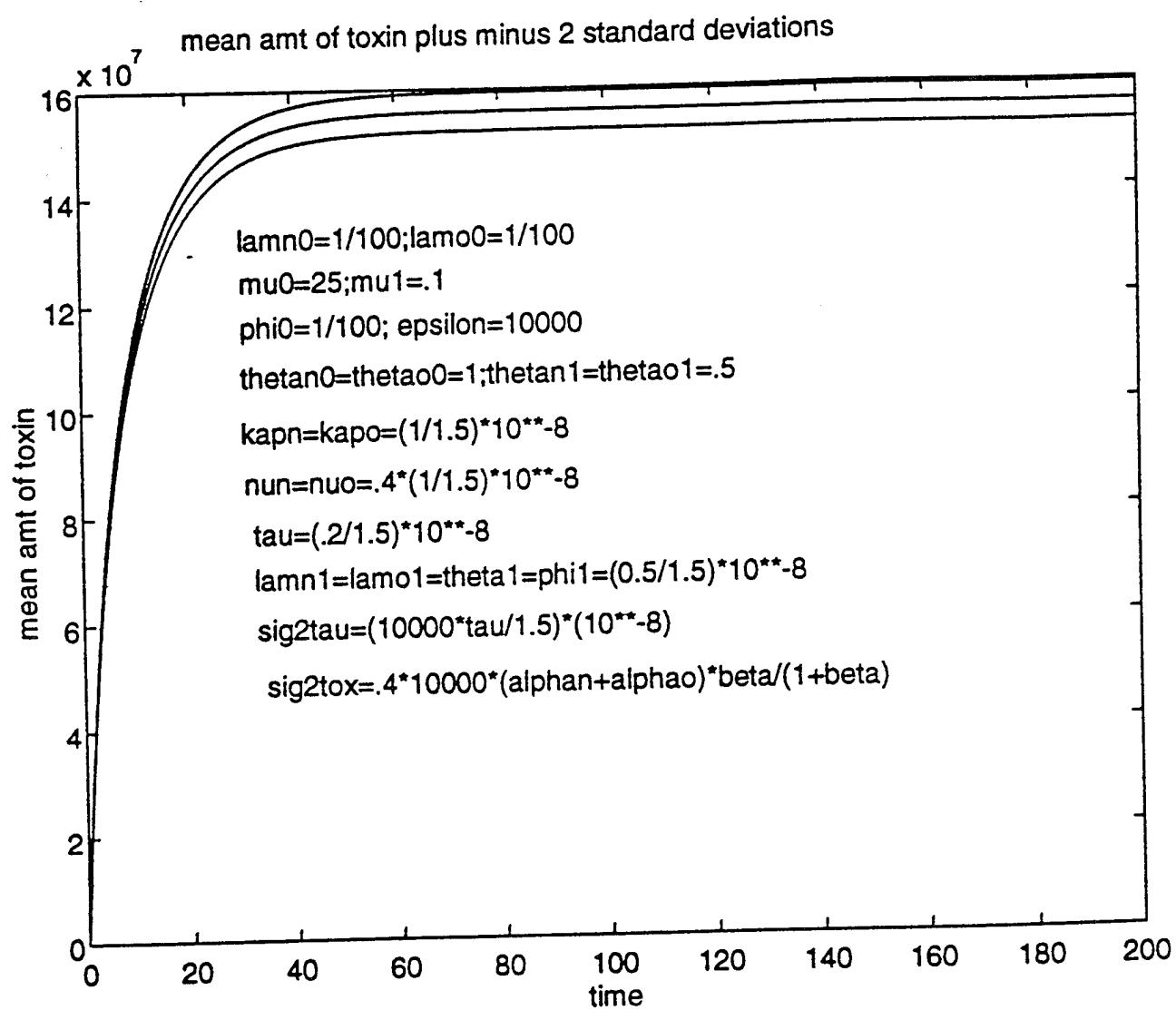


Figure 5

mean number of old cells

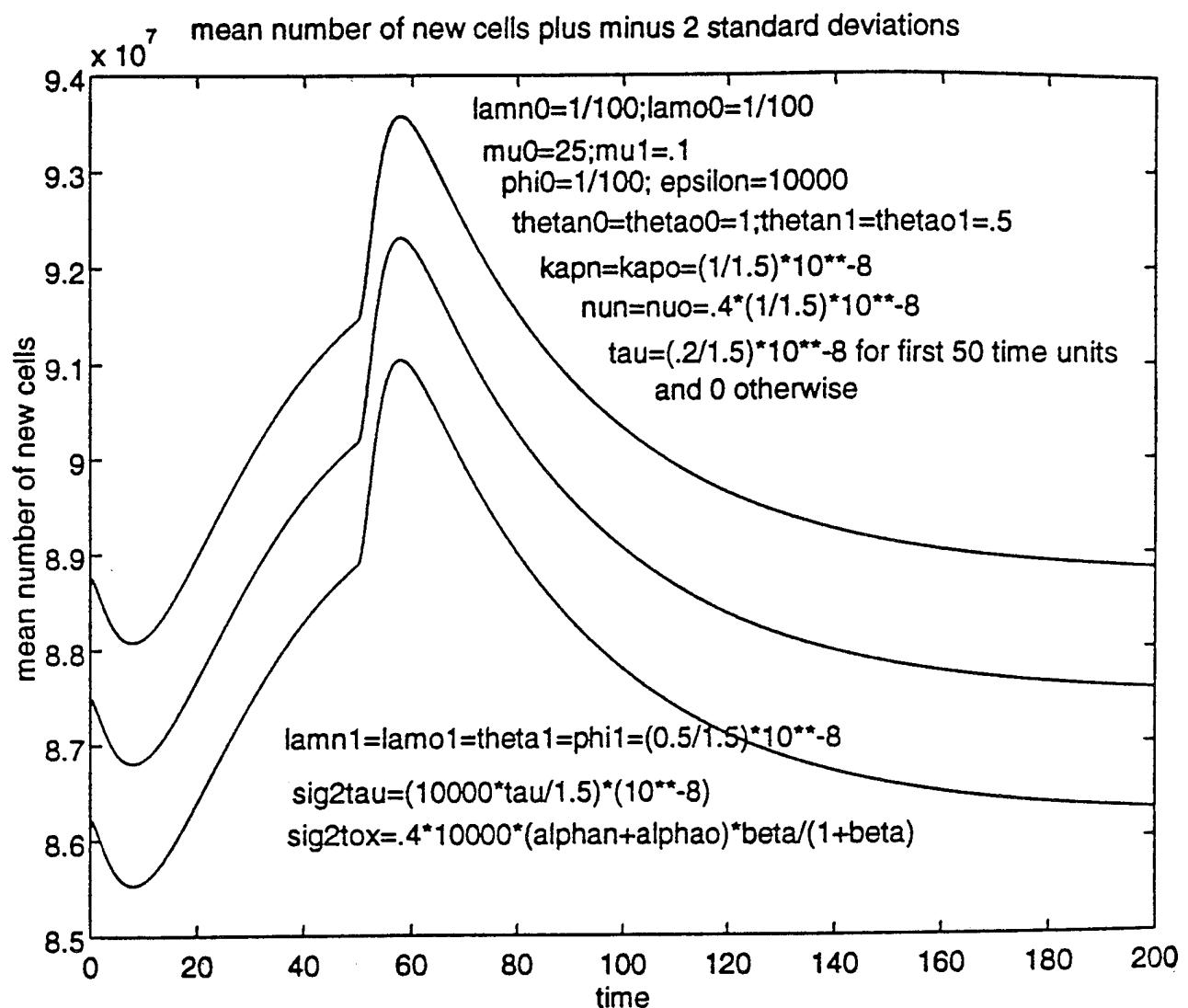


Figure 6

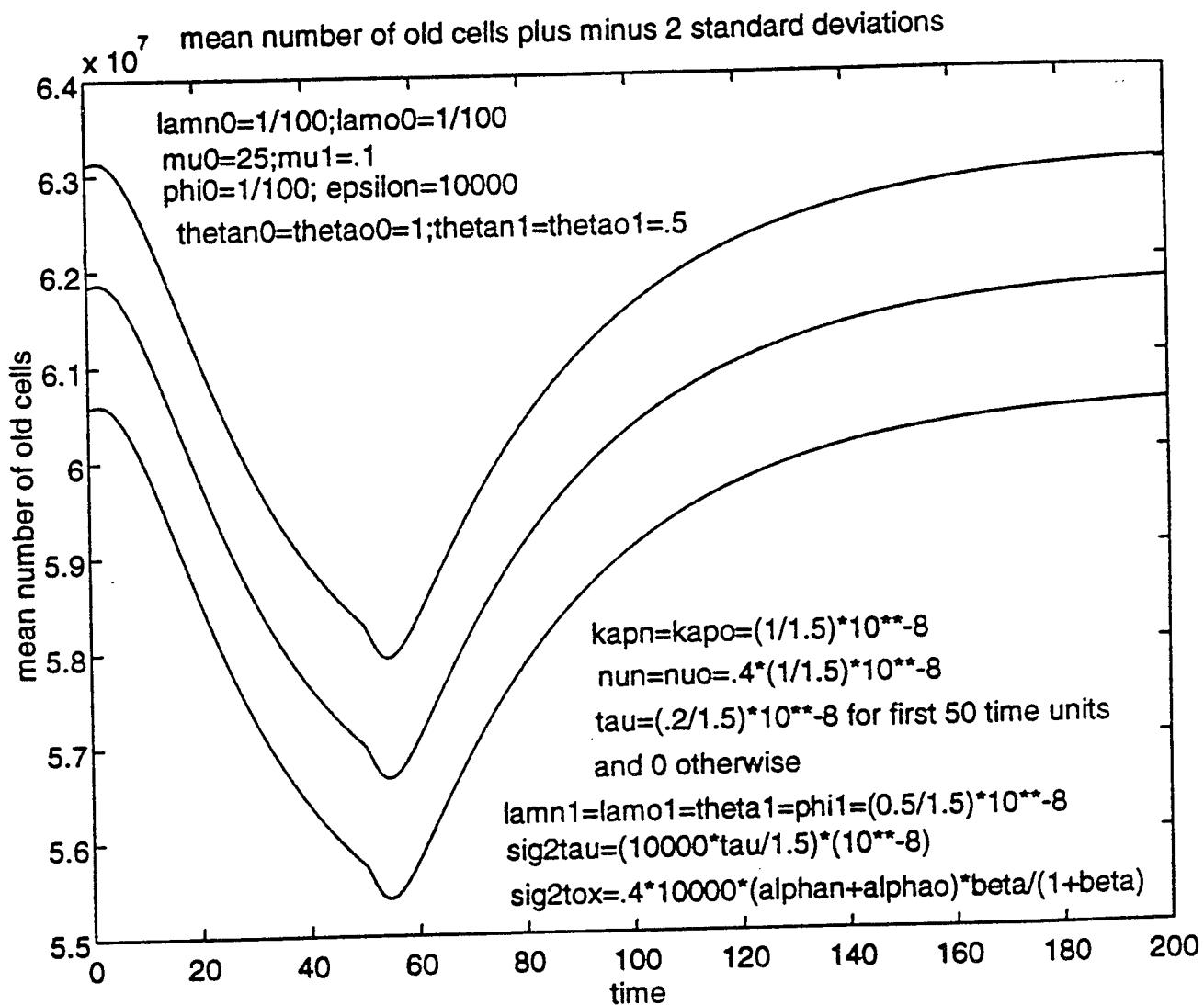


Figure 7

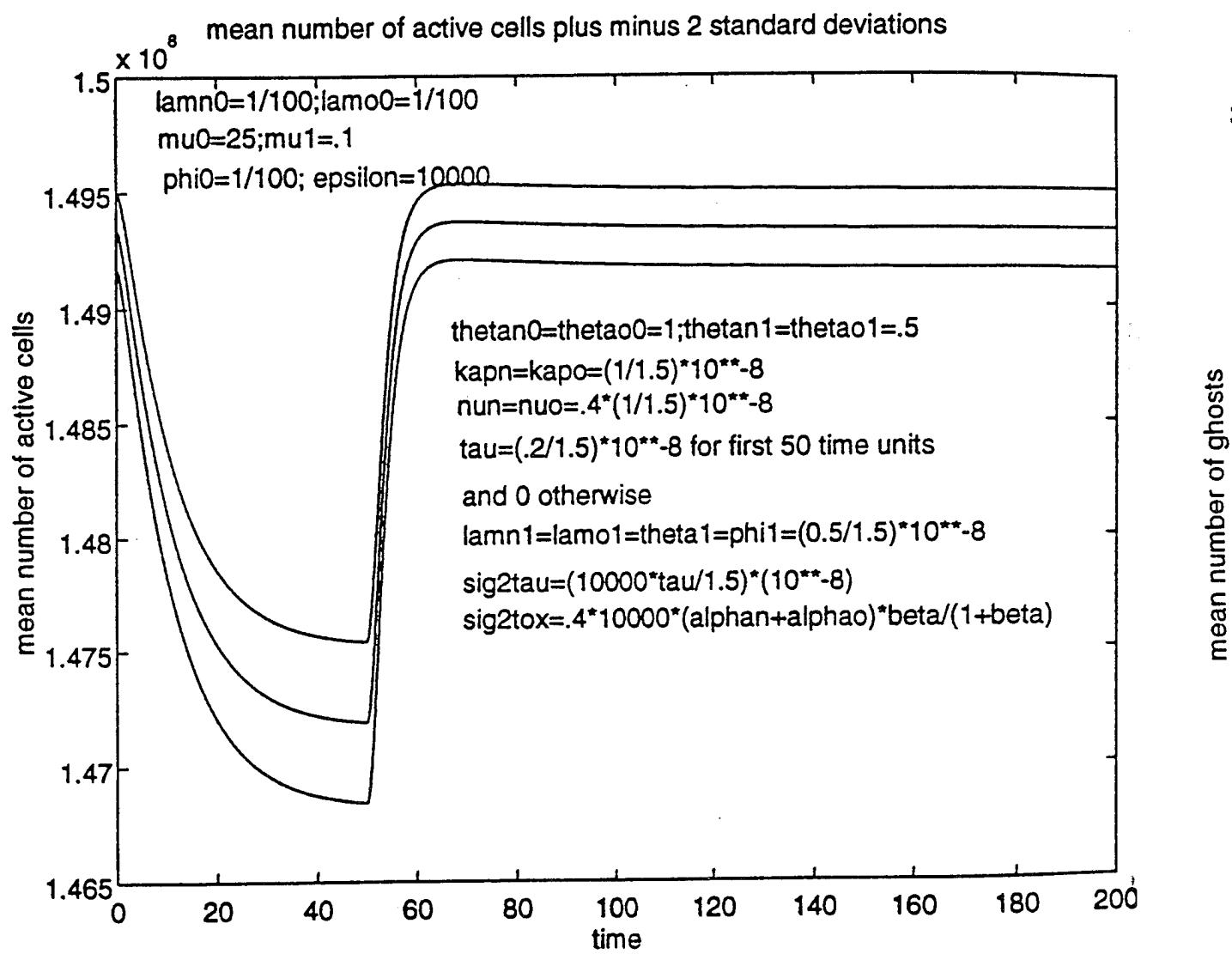


Figure 8

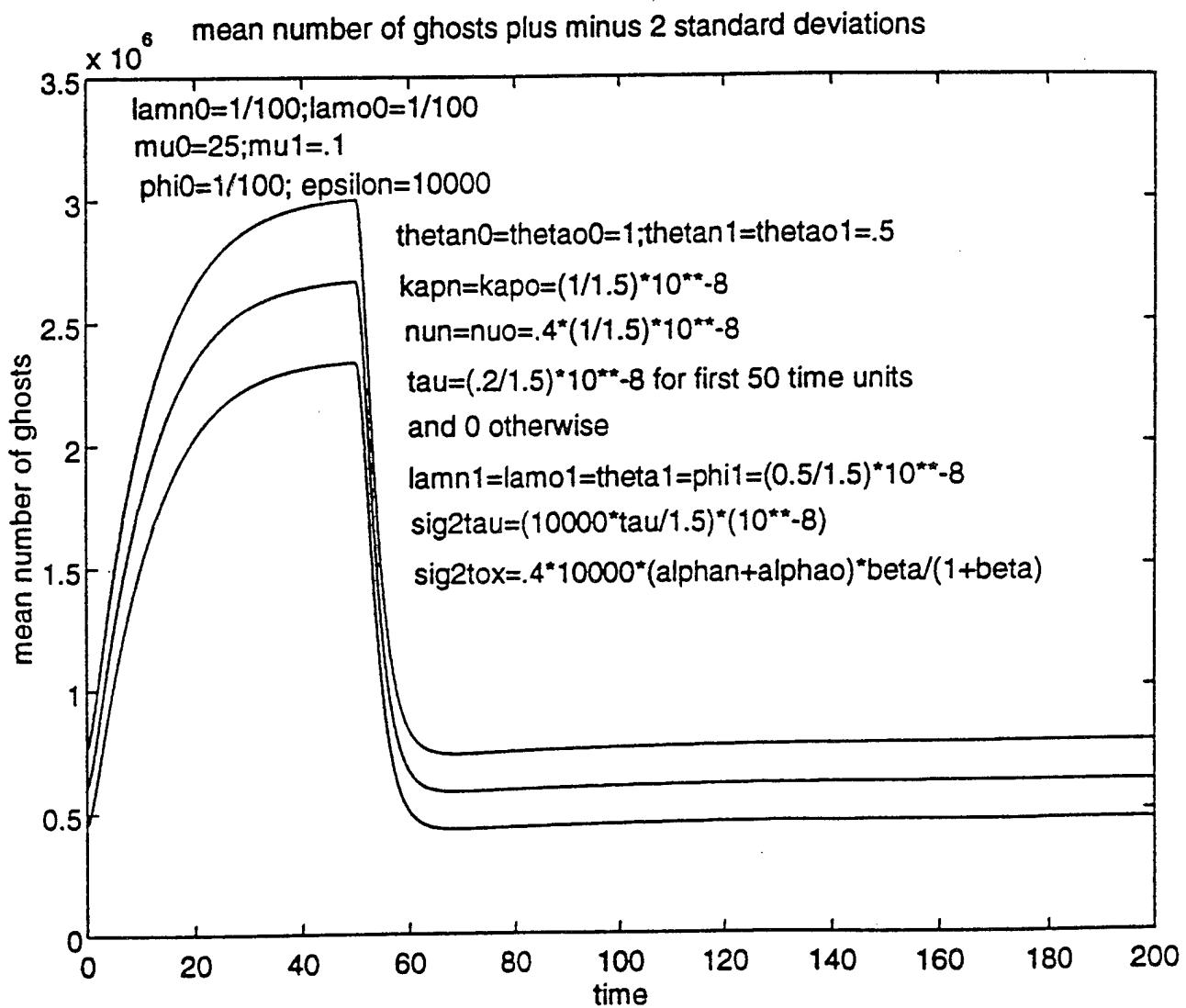


Figure 9

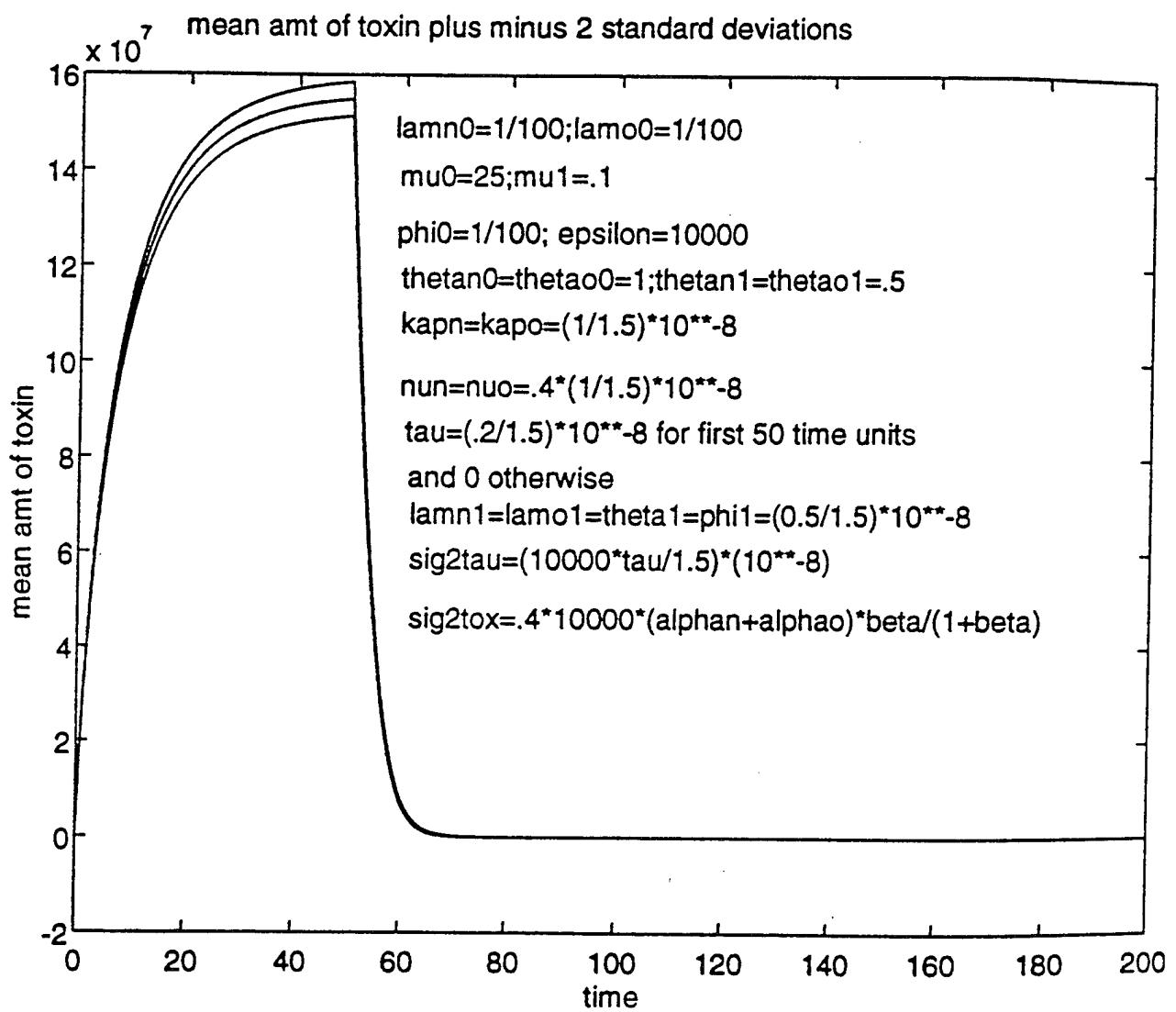


Figure 10

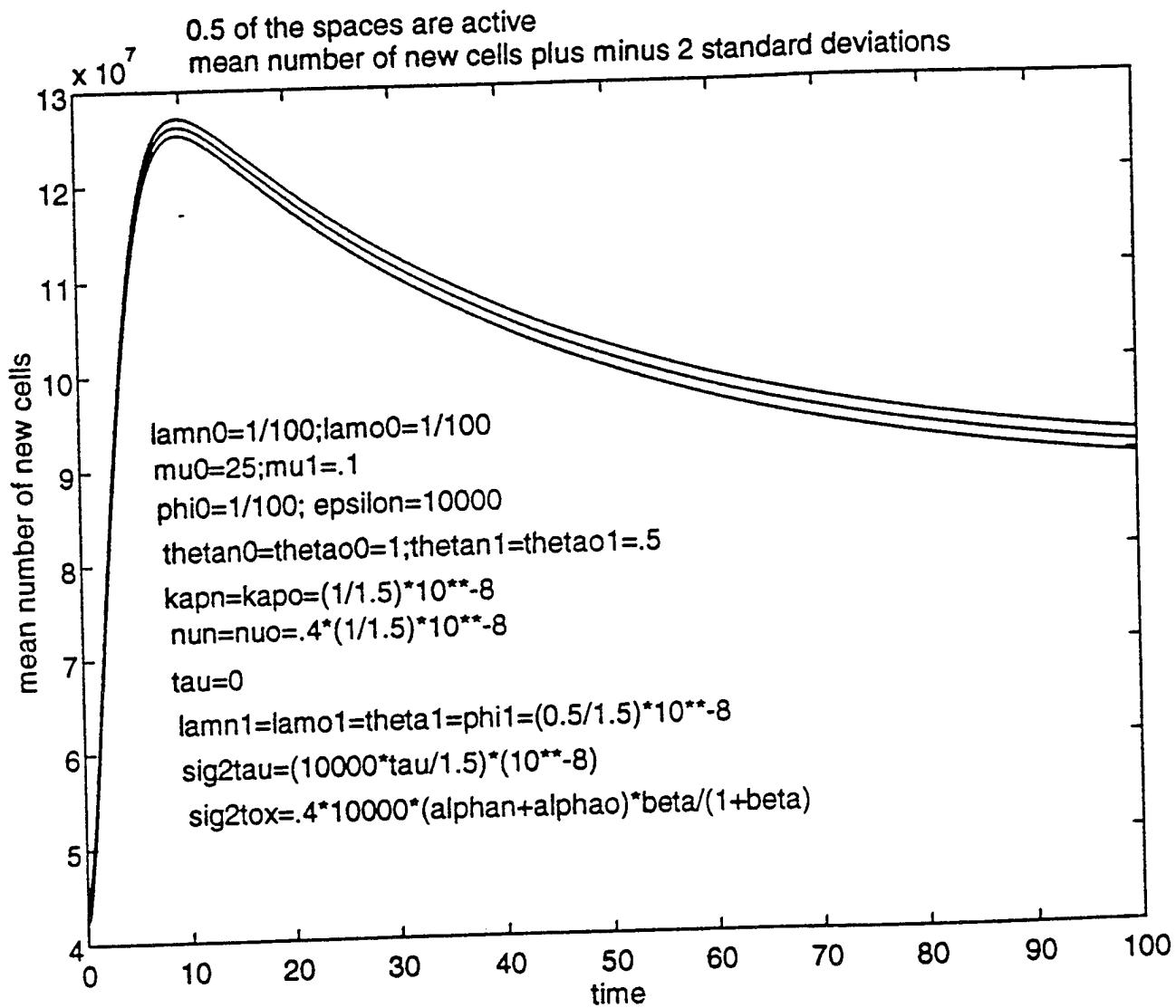


Figure 11

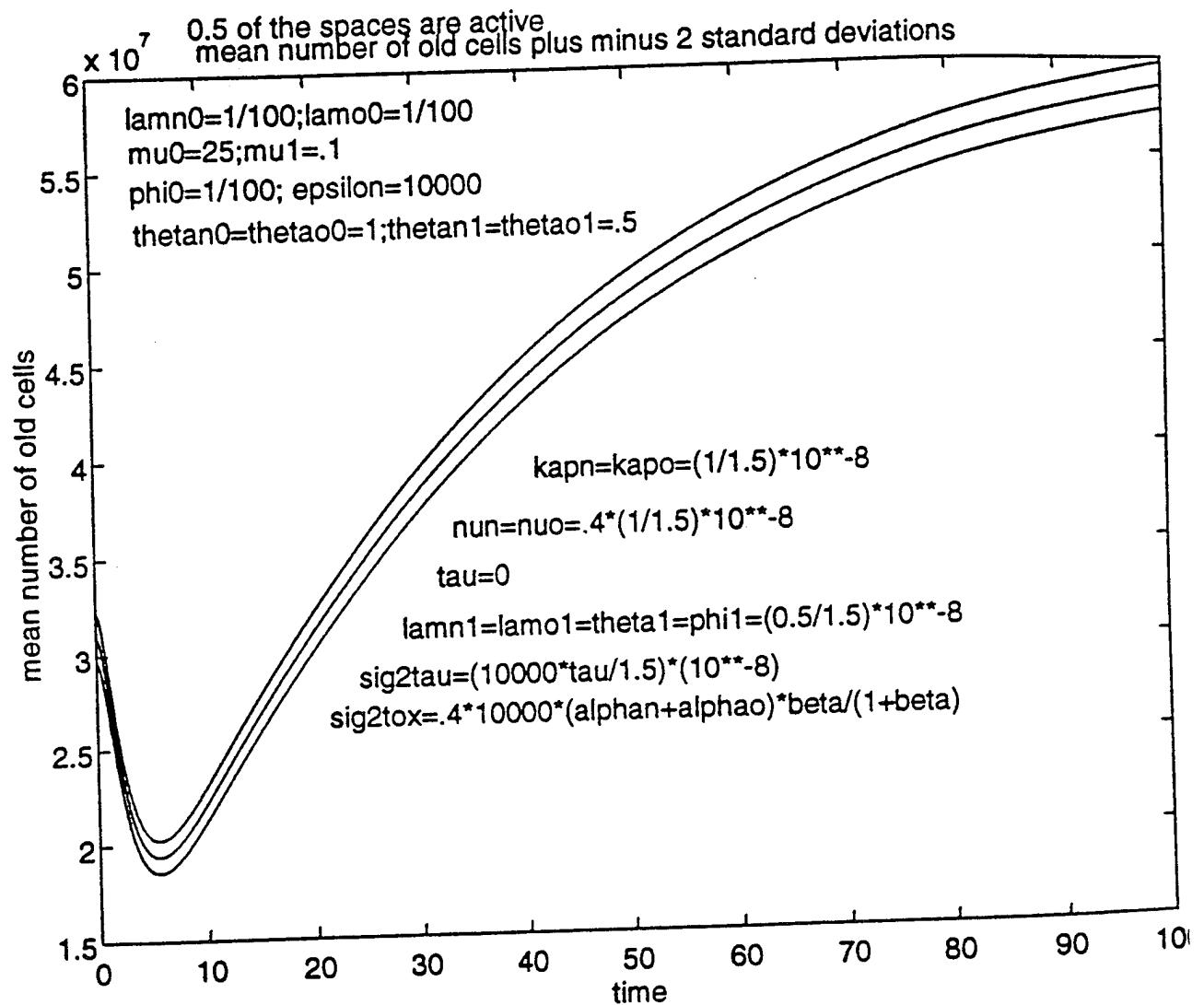


Figure 12

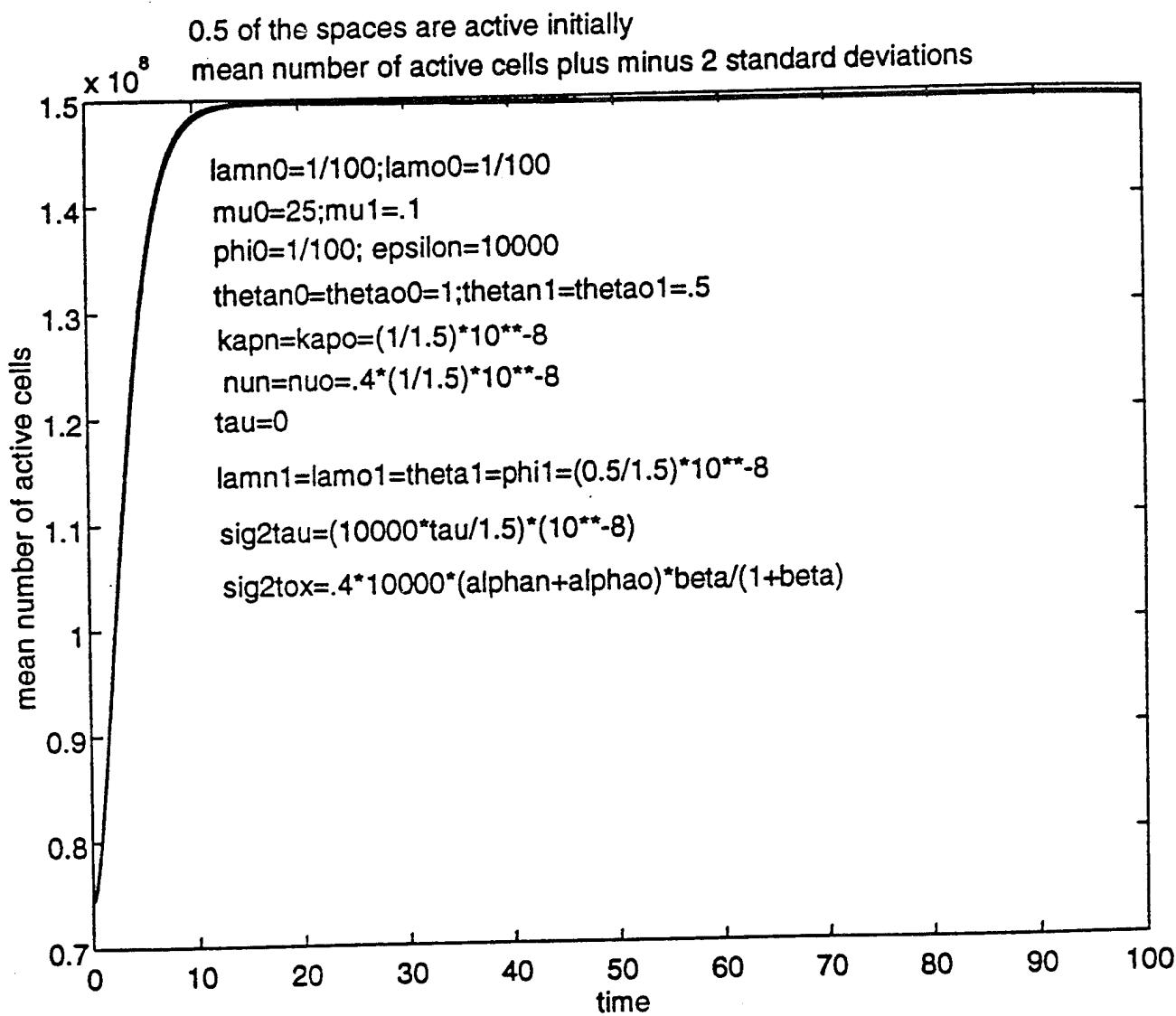


Figure 13

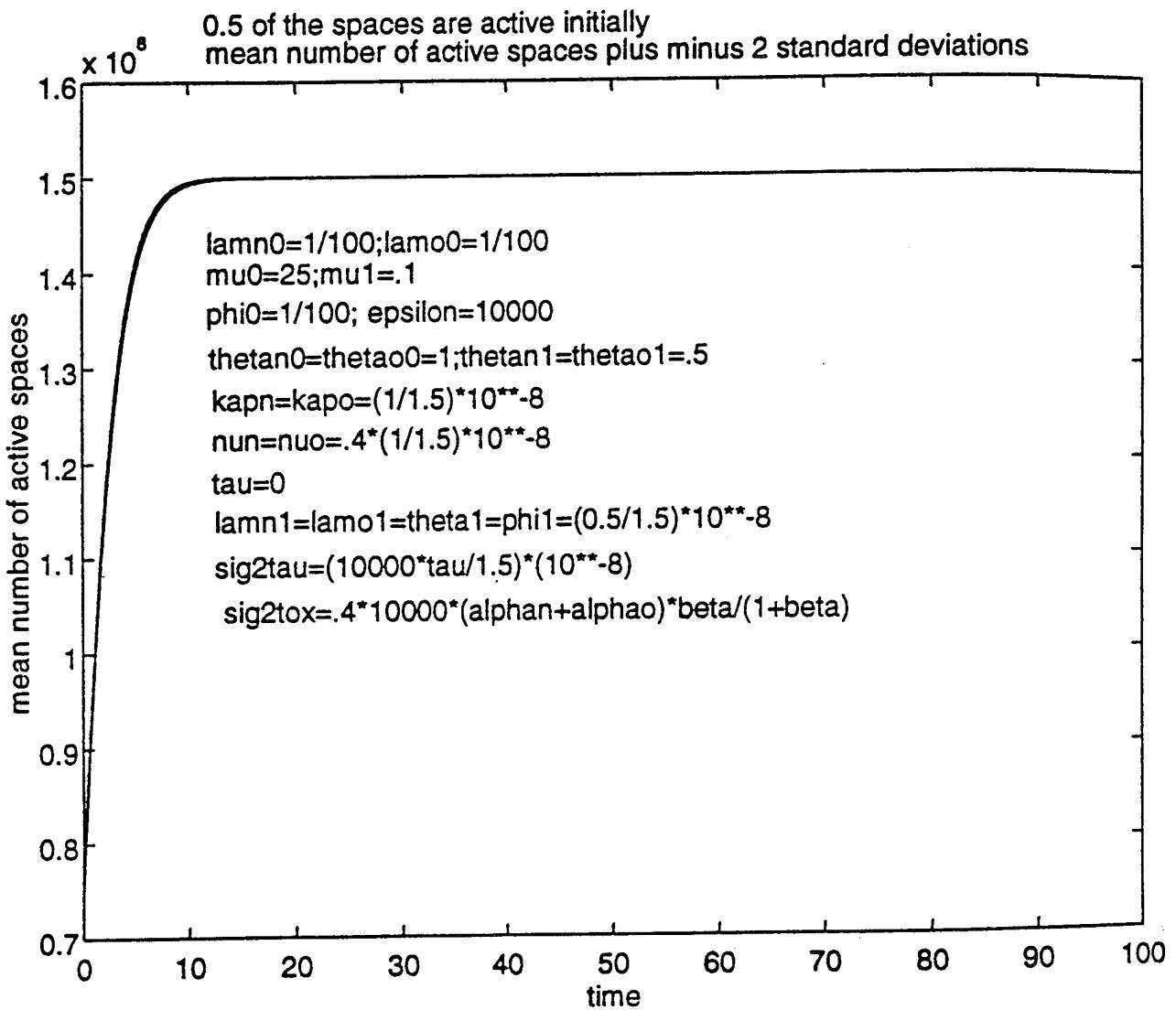


Figure 14

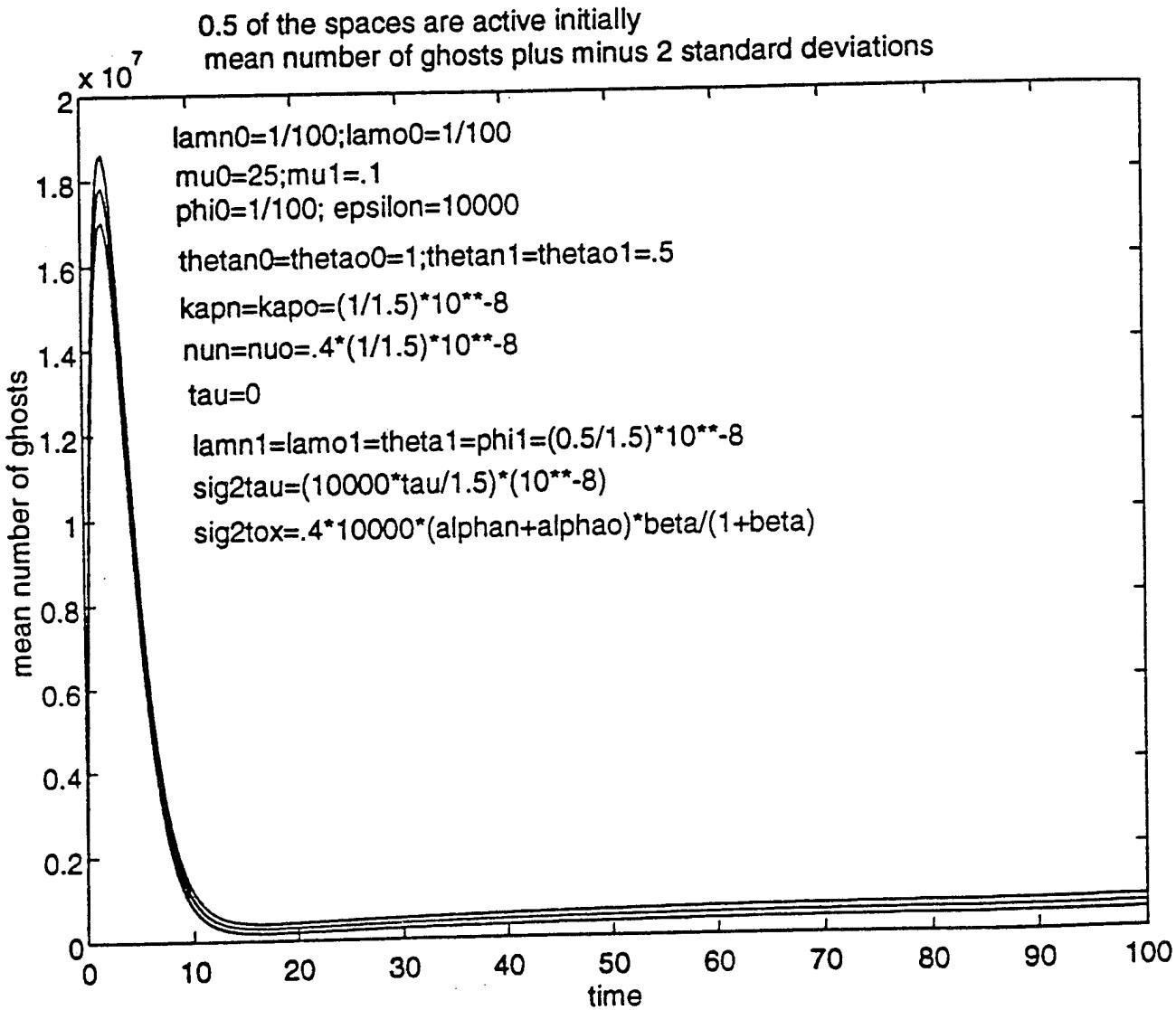


Figure 15

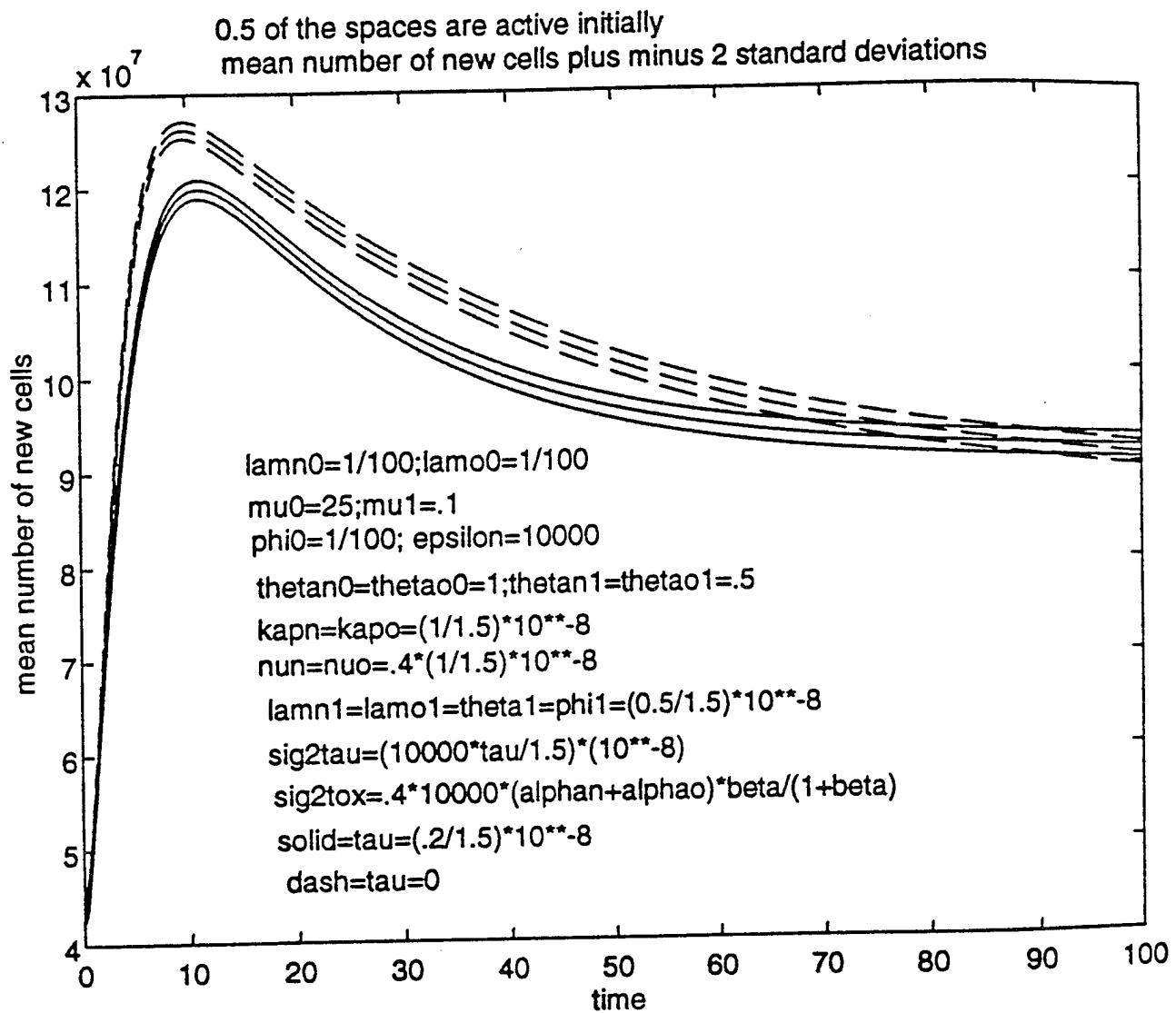


Figure 16

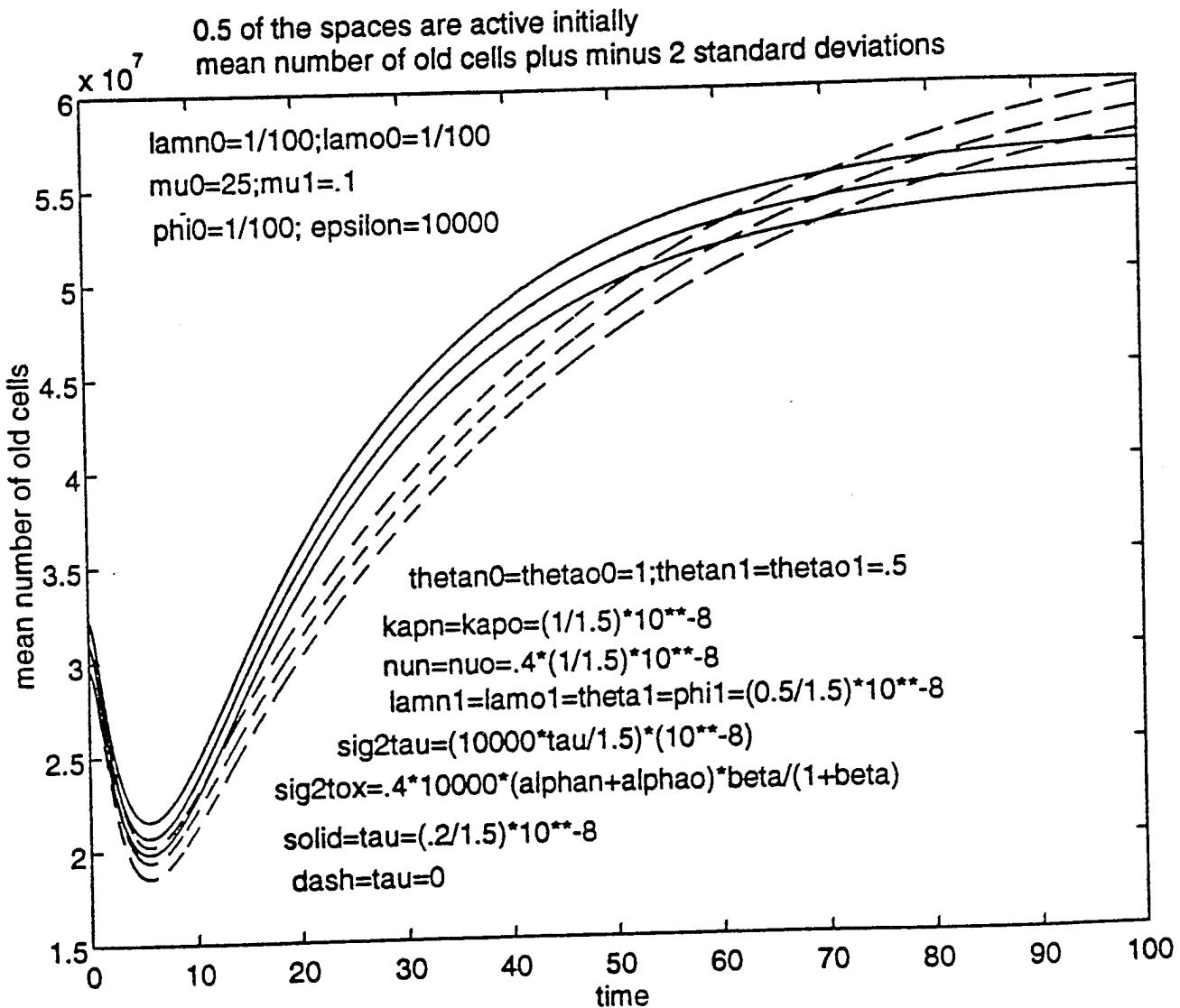


Figure 17

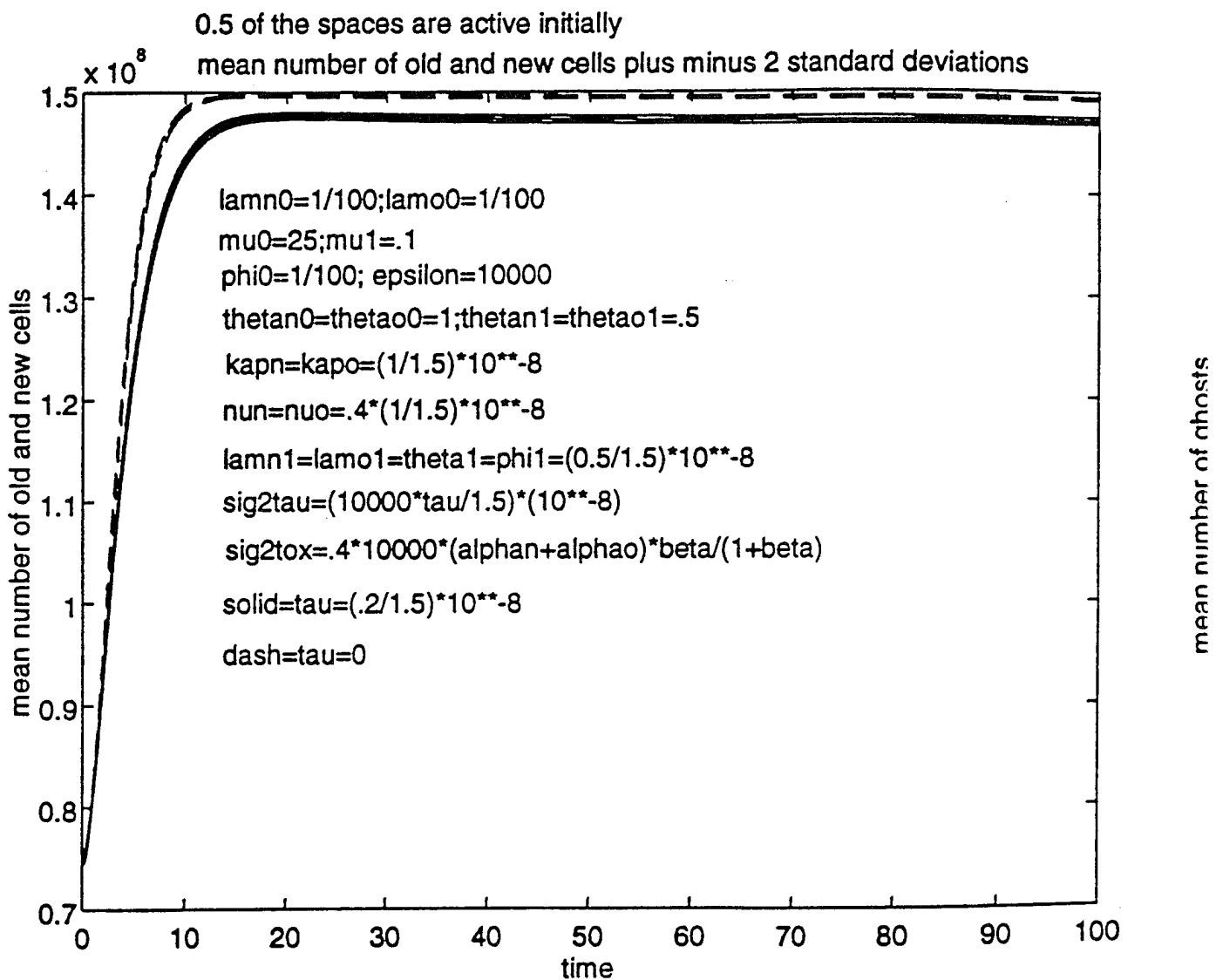


Figure 18

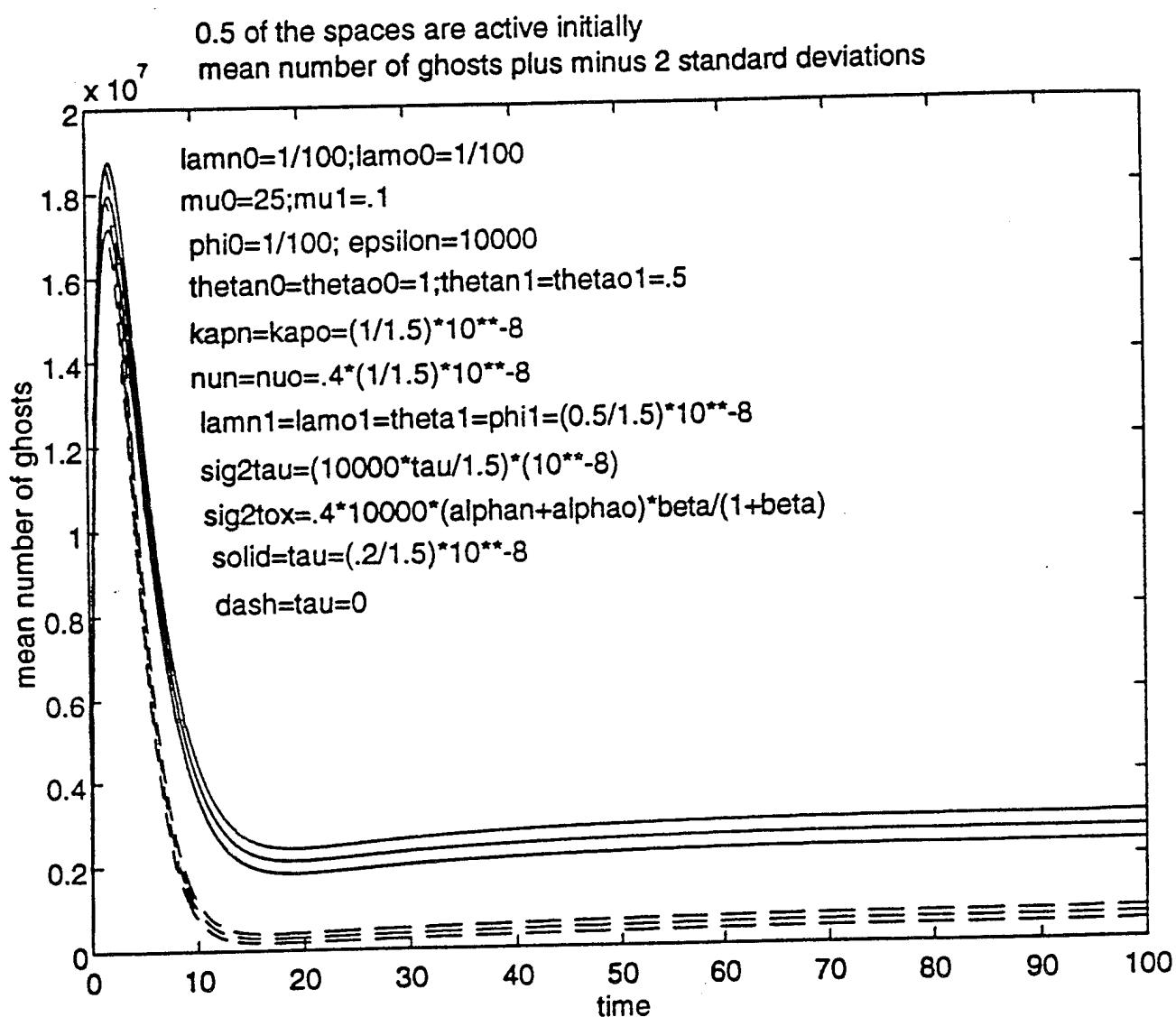


Figure 19

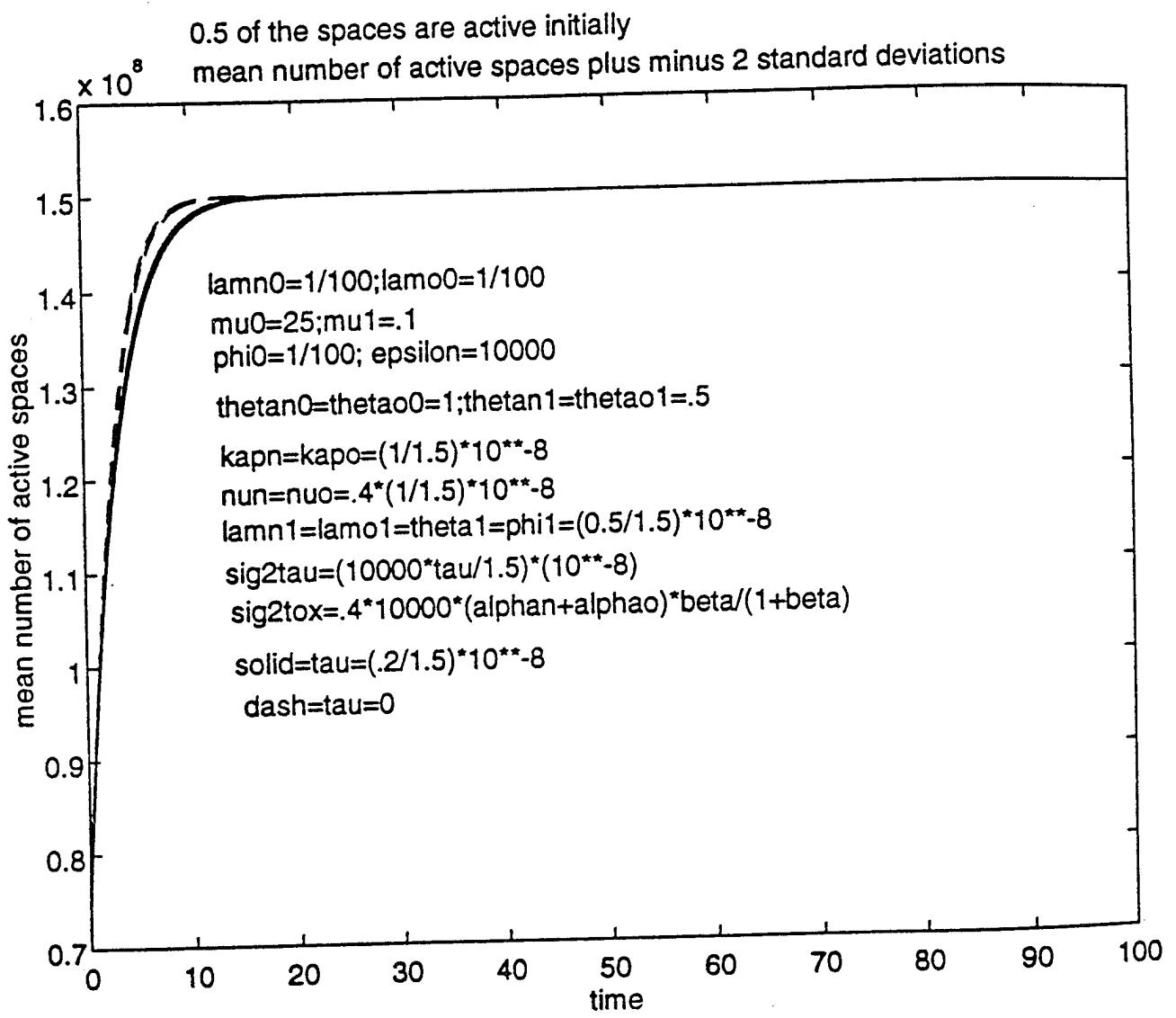


Figure 20

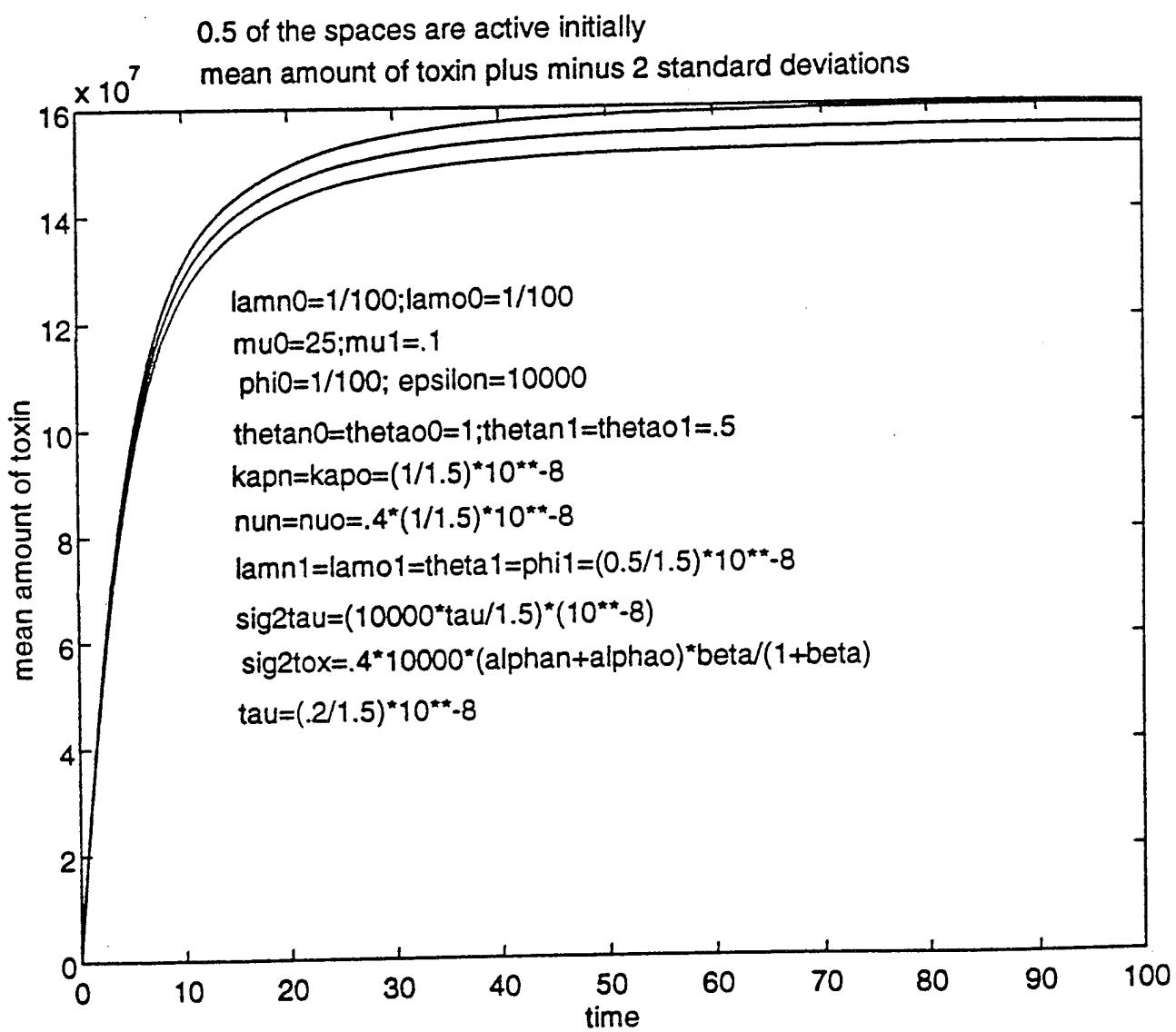


Figure 21

Appendix 1

This appendix provides the detailed asymptotic development of both the deterministic approximation (5.5) – (5.9) but also for the stochastic components introduced in (6.7). Start by introducing the normalization (6.7) into (6.1) – (6.5) and divide by C_0 . The following results are obtained:

$$\begin{aligned}
 d\alpha_n(t) + \frac{1}{\sqrt{C_0}} dX_n(t) = & - \left[\lambda_n(\beta(t)) + \lambda'_n(\beta(t)) \frac{Y(t)}{\sqrt{C_0}} \right] \left[\alpha_n(t) + \frac{1}{\sqrt{C_0}} X_n(t) \right] dt \\
 & - \left[\theta_n(\beta(t)) + \theta'_n(\beta(t)) \frac{Y(t)}{\sqrt{C_0}} \right] \gamma(t) + \frac{1}{\sqrt{C_0}} X_g(t) \left[\frac{\alpha_n(t) + \frac{1}{\sqrt{C_0}} X_n(t)}{\eta(t) + \frac{1}{\sqrt{C_0}} X_c(t)} \right] dt \\
 & - \left[\phi(\beta(t)) + \phi'(\beta(t)) \frac{Y(t)}{\sqrt{C_0}} \right] \left[\alpha_n(t) + \frac{1}{\sqrt{C_0}} X_n(t) \right] dt \\
 & + \left[\mu(\beta(t)) + \mu'(\beta(t)) \frac{Y(t)}{\sqrt{C_0}} \right] \left\{ [\eta(t) - \alpha_n(t) - \alpha_o(t) - \gamma(t)] \right. \\
 & \left. + \frac{1}{\sqrt{C_0}} [X_c(t) - X_n(t) - X_o(t) - X_g(t)] \right\} dt \\
 & - \frac{1}{\sqrt{C_0}} \sqrt{\epsilon \lambda_n(\beta(t)) \alpha_n(t)} dW_{\lambda_n}(t) - \frac{1}{\sqrt{C_0}} \sqrt{\epsilon \phi(\beta(t)) \alpha_n(t)} dW_\phi(t) \quad (\text{A.1}) \\
 & - \frac{1}{\sqrt{C_0}} \sqrt{\epsilon \theta_n(\beta(t)) \gamma(t) \frac{\alpha_n(t)}{\eta(t)}} dW_{\theta_n}(t) \\
 & + \frac{1}{\sqrt{C_0}} \sqrt{\epsilon 4 \mu(\beta(t)) (\eta(t) - \alpha_n(t) - \alpha_o(t) - \gamma(t)) \frac{1}{2}} dW_\mu(t) + o\left(\frac{1}{\sqrt{C_0}}\right)
 \end{aligned}$$

$$\begin{aligned}
d\alpha_o(t) + \frac{1}{\sqrt{C_0}} dX_o(t) = & - \left[\lambda_o(\beta(t)) + \lambda'_o(\beta(t)) \frac{Y(t)}{\sqrt{C_0}} \right] \alpha_o(t) + \frac{1}{\sqrt{C_0}} X_o(t) dt \\
& - \left[\theta_o(\beta(t)) + \theta'_o(\beta(t)) \frac{Y(t)}{\sqrt{C_0}} \right] \gamma(t) + \frac{1}{\sqrt{C_0}} X_g(t) \left[\frac{\alpha_o(t) + \frac{1}{\sqrt{C_0}} X_o(t)}{\eta(t) + \frac{1}{\sqrt{C_0}} X_c(t)} \right] dt \\
& + \left[\phi(\beta(t)) + \phi'(\beta(t)) \frac{Y(t)}{\sqrt{C_0}} \right] \alpha_n(t) + \frac{1}{\sqrt{C_0}} X_n(t) dt \quad (\text{A.2}) \\
& - \frac{1}{\sqrt{C_0}} \sqrt{\varepsilon \lambda_o(\beta(t)) \alpha_o(t)} dW_{\lambda_o}(t) + \frac{1}{\sqrt{C_0}} \sqrt{\varepsilon \phi(\beta(t)) \alpha_n(t)} dW_\phi(t) \\
& - \frac{1}{\sqrt{C_0}} \sqrt{\varepsilon \theta_o(\beta(t)) \gamma(t)} \frac{\alpha_o(t)}{\eta(t)} dW_{\theta_o}(t) + o\left(\frac{1}{\sqrt{C_0}}\right)
\end{aligned}$$

$$\begin{aligned}
d\gamma(t) + \frac{1}{\sqrt{C_0}} dX_g(t) = & \\
& - \left[\theta_n(\beta(t)) + \theta'_n(\beta(t)) \frac{Y(t)}{\sqrt{C_0}} \right] \left[\gamma(t) + \frac{1}{\sqrt{C_0}} X_g(t) \right] \left[\frac{\alpha_n(t) + \frac{1}{\sqrt{C_0}} X_n(t)}{\eta(t) + \frac{1}{\sqrt{C_0}} X_c(t)} \right] dt \\
& - \left[\theta_o(\beta(t)) + \theta'_o(\beta(t)) \frac{Y(t)}{\sqrt{C_0}} \right] \left[\gamma(t) + \frac{1}{\sqrt{C_0}} X_g(t) \right] \left[\frac{\alpha_o(t) + \frac{1}{\sqrt{C_0}} X_o(t)}{\eta(t) + \frac{1}{\sqrt{C_0}} X_c(t)} \right] dt \\
& + \left[\xi_n(\beta(t)) + \xi'_n(\beta(t)) \frac{Y(t)}{\sqrt{C_0}} \right] \left[\alpha_n(t) + \frac{1}{\sqrt{C_0}} X_n(t) \right] \left[1 - \eta(t) - \frac{1}{\sqrt{C_0}} X_c(t) \right] dt \\
& + \left[\xi_o(\beta(t)) + \xi'_o(\beta(t)) \frac{Y(t)}{\sqrt{C_0}} \right] \left[\alpha_o(t) + \frac{1}{\sqrt{C_0}} X_o(t) \right] \left[1 - \eta(t) - \frac{1}{\sqrt{C_0}} X_c(t) \right] dt \\
& + \left[\lambda_o(\beta(t)) + \lambda'_o(\beta(t)) \frac{Y(t)}{\sqrt{C_0}} \right] \left[\alpha_o(t) + \frac{1}{\sqrt{C_0}} X_o(t) \right] dt \\
& + \left[\lambda_n(\beta(t)) + \lambda'_n(\beta(t)) \frac{Y(t)}{\sqrt{C_0}} \right] \left[\alpha_n(t) + \frac{1}{\sqrt{C_0}} X_n(t) \right] dt \\
& - \frac{1}{\sqrt{C_0}} \sqrt{\varepsilon \theta_n(\beta(t)) \gamma(t) \alpha_n(t) / \eta(t)} dW_{\theta_n}(t) \\
& - \frac{1}{\sqrt{C_0}} \sqrt{\varepsilon \theta_o(\beta(t)) \gamma(t) \alpha_o(t) / \eta(t)} dW_{\theta_o}(t) \\
& + \frac{1}{\sqrt{C_0}} \sqrt{\varepsilon \lambda_o(\beta(t)) \alpha_o(t)} dW_{\lambda_o}(t) + \frac{1}{\sqrt{C_0}} \sqrt{\varepsilon \lambda_n(\beta(t)) \alpha_n(t)} dW_{\lambda_n}(t) \\
& + \frac{1}{\sqrt{C_0}} \sqrt{\varepsilon \xi_n(\beta(t)) \alpha_n(t) [1 - \eta(t)]} dW_{\xi_n}(t) \\
& + \frac{1}{\sqrt{C_0}} \sqrt{\varepsilon \xi_o(\beta(t)) \alpha_o(t) [1 - \eta(t)]} dW_{\xi_o}(t) + o\left(\frac{1}{\sqrt{C_0}}\right)
\end{aligned} \tag{A.3}$$

$$\begin{aligned}
d\eta(t) + \frac{1}{\sqrt{C_0}} dX_c(t) = & \\
& \left[\xi_o(\beta(t)) + \xi'_o(\beta(t)) \frac{1}{\sqrt{C_0}} Y(t) \right] \left[\alpha_o(t) + \frac{1}{\sqrt{C_0}} X_o(t) \right] \left[1 - \eta(t) - \frac{1}{\sqrt{C_0}} X_c(t) \right] dt \\
& + \left[\xi_n(\beta(t)) + \xi'_n(\beta(t)) \frac{1}{\sqrt{C_0}} Y(t) \right] \left[\alpha_n(t) + \frac{1}{\sqrt{C_0}} X_n(t) \right] \left[1 - \eta(t) - \frac{1}{\sqrt{C_0}} X_c(t) \right] dt \quad (\text{A.4}) \\
& + \frac{1}{\sqrt{C_0}} \sqrt{\varepsilon \xi_o(\beta(t)) \alpha_o(t) [1 - \eta(t)]} dW_{\xi_o}(t) \\
& + \frac{1}{\sqrt{C_0}} \sqrt{\varepsilon \xi_n(\beta(t)) \alpha_n(t) [1 - \eta(t)]} dW_{\xi_n}(t) + o\left(\frac{1}{\sqrt{C_0}}\right)
\end{aligned}$$

$$\begin{aligned}
\frac{d\beta(t)}{dt} + \frac{1}{\sqrt{C_0}} \frac{dY(t)}{dt} = & \delta(\alpha_n(t), \alpha_o(t), \beta(t), \tau(t)) \\
& + \delta'_\beta(\alpha_n(t), \alpha_o(t), \beta(t), \tau(t)) \frac{Y(t)}{\sqrt{C_0}} \\
& + \delta'_{\alpha_n}(\alpha_n(t), \alpha_o(t), \beta(t), \tau(t)) \frac{X_n(t)}{\sqrt{C_0}} \\
& + \delta'_{\alpha_o}(\alpha_n(t), \alpha_o(t), \beta(t), \tau(t)) \frac{X_o(t)}{\sqrt{C_0}} \quad (\text{A.5}) \\
& + \frac{1}{\sqrt{C_0}} \sigma_T(\beta(t), \alpha_n(t), \alpha_o(t), \tau(t)) dW_{\sigma_T}(t) \\
& + \frac{1}{\sqrt{C_0}} \sigma_\tau dW_\tau(t) + o\left(\frac{1}{\sqrt{C_0}}\right)
\end{aligned}$$

The terms of order 1 in (A.1) – (A.5) yield the deterministic equations (5.5) – (5.9).

The terms of order $1/\sqrt{C_0}$ in (A.1) – (A.5) give the following stochastic differential equations for the noise terms.

$$\begin{aligned}
\frac{dX_n(t)}{dt} = & -\lambda'_n(\beta(t))\alpha_n(t)Y(t) - \lambda_n(\beta(t))X_n(t) \\
& -\theta_n(\beta(t))X_g(t)\frac{\alpha_n(t)}{\eta(t)} - \theta'_n(\beta(t))\gamma(t)\frac{\alpha_n(t)}{\eta(t)}Y(t) \\
& -\theta_n(\beta(t))\gamma(t)\left[\frac{1}{\eta(t)}X_n(t) - \frac{\alpha_n(t)}{\eta(t)^2}X_c(t)\right] \\
& -\phi(\beta(t))X_n(t) - \phi'(\beta(t))\alpha_n(t)Y(t) \\
& +\mu(\beta(t))[X_c(t) - X_n(t) - X_o(t) - X_g(t)] \\
& +\mu'(\beta(t))[\eta(t) - \alpha_n(t) - \alpha_o(t) - \gamma(t)]Y(t) \tag{A.6} \\
& -\sqrt{\varepsilon\lambda_n(\beta(t))\alpha_n(t)}dW_{\lambda_n}(t) - \sqrt{\varepsilon\phi(\beta(t))\alpha_n(t)}dW_\phi(t) \\
& -\sqrt{\varepsilon\theta_n(\beta(t))\gamma(t)}\frac{\alpha_n(t)}{\eta(t)}dW_{\theta_n}(t) \\
& +\sqrt{\varepsilon 2\mu(\beta(t))(\eta(t) - \alpha_n(t) - \alpha_o(t) - \gamma(t))}dW_\mu(t)
\end{aligned}$$

$$\begin{aligned}
\frac{dX_o(t)}{dt} = & -\lambda_o(\beta(t))X_o(t) - \lambda'_o(\beta(t))\alpha_o(t)Y(t) \\
& -\theta_o(\beta(t))\frac{\alpha_o(t)}{\eta(t)}X_g(t) - \theta'_o(\beta(t))\gamma(t)\frac{\alpha_o(t)}{\eta(t)}Y(t) \\
& -\theta_o(\beta(t))\gamma(t)\left[\frac{1}{\eta(t)}X_o(t) - \frac{\alpha_o(t)}{\eta(t)^2}X_c(t)\right] \tag{A.7} \\
& +\phi(\beta(t))X_n(t) + \phi'(\beta(t))\alpha_n(t)Y(t) \\
& -\sqrt{\varepsilon\lambda_o(\beta(t))\alpha_o(t)}dW_{\lambda_o}(t) + \sqrt{\varepsilon\phi(\beta(t))\alpha_n(t)}dW_\phi(t) \\
& -\sqrt{\varepsilon\theta_o(\beta(t))\gamma(t)}\frac{\alpha_o(t)}{\eta(t)}dW_{\theta_o}(t)
\end{aligned}$$

$$\begin{aligned}
\frac{dX_g(t)}{dt} = & -\theta_n(\beta(t))X_g(t)\frac{\alpha_n(t)}{\eta(t)} - \theta'_n(\beta(t))\gamma(t)\frac{\alpha_n(t)}{\eta(t)}Y(t) \\
& -\theta_n(\beta(t))\gamma(t)\left[\frac{1}{\eta(t)}X_n(t) - \frac{\alpha_n(t)}{\eta(t)^2}X_c(t)\right] \\
& -\theta_o(\beta(t))X_g(t)\frac{\alpha_o(t)}{\eta(t)} - \theta'_o(\beta(t))\gamma(t)\frac{\alpha_o(t)}{\eta(t)}Y(t) \\
& -\theta_o(\beta(t))\gamma(t)\left[\frac{1}{\eta(t)}X_o(t) - \frac{\alpha_o(t)}{\eta(t)^2}X_c(t)\right] \\
& -\xi_n(\beta(t))\alpha_n(t)X_c(t) \\
& +\xi_n'(\beta(t))(1-\eta(t))X_n(t) + \xi_n'(\beta(t))\alpha_n(t)(1-\eta(t))Y(t) \\
& -\xi_o(\beta(t))\alpha_o(t)X_c(t) + \xi_o'(\beta(t))(1-\eta(t))X_o(t) \\
& +\xi_o'(\beta(t))\alpha_o(t)(1-\eta(t))Y(t) \\
& +\lambda_o(\beta(t))X_o(t) + \lambda'_o(\beta(t))\alpha_o(t)Y(t) \\
& +\lambda_n(\beta(t))X_n(t) + \lambda'_n(\beta(t))\alpha_n(t)Y(t) \\
& -\sqrt{\varepsilon\theta_n(\beta(t))\gamma(t)\alpha_n(t)/\eta(t)}dW_{\theta_n}(t) - \sqrt{\varepsilon\theta_o(\beta(t))\gamma(t)\alpha_o(t)/\eta(t)}dW_{\theta_o}(t) \\
& +\sqrt{\varepsilon\lambda_o(\beta(t))\alpha_o(t)}dW_{\lambda_o}(t) + \sqrt{\varepsilon\lambda_n(\beta(t))\alpha_n(t)}dW_{\lambda_n}(t) \\
& +\sqrt{\varepsilon\xi_n(\beta(t))\alpha_n(t)[1-\eta(t)]}dW_{\xi_n}(t) + \sqrt{\varepsilon\xi_o(\beta(t))\alpha_o(t)[1-\eta(t)]}dW_{\xi_o}(t)
\end{aligned} \tag{A.8}$$

$$\begin{aligned}
\frac{dX_c(t)}{dt} = & -\xi_o(\beta(t))\alpha_o(t)X_c(t) + \xi'_o(\beta(t))\alpha_o(t)[1-\eta(t)]Y(t) \\
& + \xi_o(\beta(t))[1-\eta(t)]X_o(t) \\
& - \xi_n(\beta(t))\alpha_n(t)X_c(t) + \xi'_n(\beta(t))\alpha_n(t)[1-\eta(t)]Y(t) \\
& + \xi_n(\beta(t))[1-\eta(t)]X_n(t)
\end{aligned} \tag{A.9}$$

$$\begin{aligned}
\frac{dY(t)}{dt} = & \delta'_\beta(\beta(t), \alpha_n(t), \alpha_o(t), \tau(t))Y(t) \\
& + \delta'_{\alpha_n}(\beta(t), \alpha_n(t), \alpha_o(t), \tau(t))X_n(t) \\
& + \delta'_{\alpha_o}(\beta(t), \alpha_n(t), \alpha_o(t), \tau(t))X_o(t) \\
& + \sigma_T(\beta(t), \alpha_n(t), \alpha_o(t), \tau(t))dW_{\sigma_T}(t) \\
& + \sigma_\tau dW_\tau(t)
\end{aligned} \tag{A.10}$$

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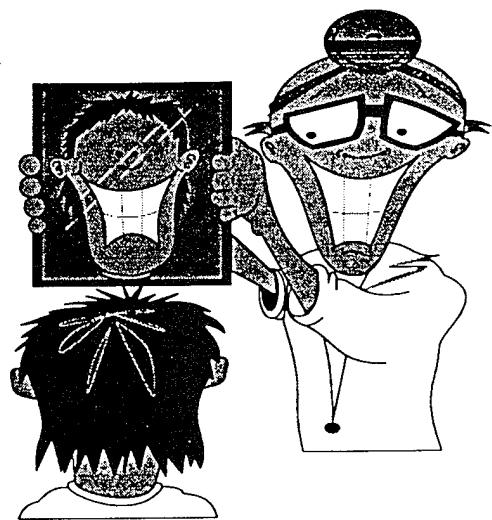
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Publications for 1995

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**Naval Dental Research Institute
Detachment
Bethesda, MD**



ORAL MICROBIOLOGY

GLENN A. MILLER, PH.D.

MICROBIOLOGY
1995-1996

Lecture	Date	Time	Subject
1	8/24	0730-0820	Bacterial structure, classification, and identification
2	8/24	0830-0920	Bacterial physiology and metabolism
3	8/31	0730-0820	Introduction to virology
4	8/31	0830-0920	Viruses of dental interest
5	9/7	0730-0820	Bacterial physiology and metabolism, cont.
6	9/7	0830-0920	Oral microbial ecology
7	9/20	0930-1020	Endogenous bacterial flora
8	9/20	1030-1120	Host parasite interactions
9	9/21	0730-0820	Mechanisms of oral colonization and the microbiology of dental plaque
10	9/21	0830-0920	Mechanisms of oral colonization and the microbiology of dental plaque, cont.
11	9/25	0730-0820	Microbiology of dental caries
12	9/25	0830-0920	Microbiology of periodontal disease
13	9/28	0730-0820	Microbiology of pulp and periapical infections
14	9/28	0830-0920	Pathogenic potential of the normal oral flora
15	10/4	0930-1020	Microbial genetics and genetic engineering
16	10/4	1030-1120	Antimicrobial drugs- Mechanisms of action and drug resistance

ORAL MICROBIOLOGY

1995

COURSE GOALS: Information about and understanding of the microbiology of the human oral cavity have increased rapidly in the past decade and show little sign of diminishing. Recent advances in microbiology have been pivotal in developing more rapid and more effective means of diagnosis, treatment, and prevention of dental caries, periodontal diseases, and other oral infections. In addition, AIDS, hepatitis, and other medical infections have assumed extreme importance in a dental practice setting. Also, we are currently experiencing a resurgence in the levels of pathogenicity of bacteria previously thought to be in control.

The purpose of this course is to provide Dental Residents with an understanding of contemporary oral microbiology as a basis for understanding and controlling their growth in clinical situations. This will be done with the use of a "mini-textbook" format which includes extensive written material and by expanding and elaborating on this material with in-class discussion. It will be to your advantage to read through the appropriate section prior to its discussion in class.

Grading: At the conclusion of the course an examination will be provided. The examination will be oral and will be taken in a session with other members of your speciality. Typical questions given during the examination will be taken from a group of such questions included in your handout. The specifics for the questions will be taken from the lecture notes included herein.

Grades for performance in the oral examination will be given at the completion of the exam.

In general:

A: Excellent

B: Solid performance. Complete answers to most questions

D: Poor and incomplete responses.

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LECTURE 1: BACTERIAL STRUCTURE, CLASSIFICATION, AND IDENTIFICATION

I. Prokaryotes, Eukaryotes, and Viruses

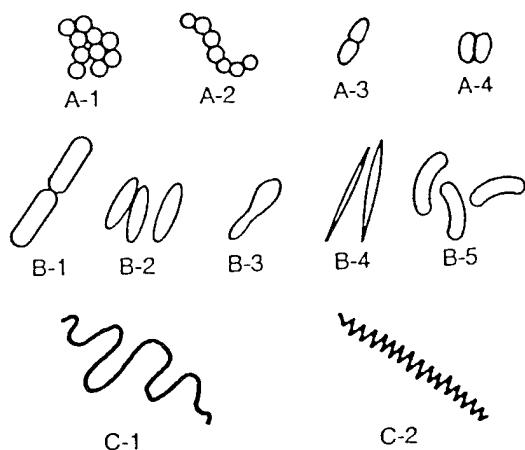
Cells have evolved into 2 fundamentally different types, **eukaryotic** and **prokaryotic**, which can be distinguished on the basis of their structure and the complexity of their organization. Fungi and protozoa are eukaryotic, whereas bacteria are prokaryotic. The eukaryotic cell has a true nucleus with multiple chromosomes surrounded by a nuclear membrane and uses a mitotic apparatus to ensure equal allocation of the chromosomes to progeny cells. The **nucleoid** of a prokaryotic cell consists of a single circular molecule of loosely organized DNA lacking a nuclear membrane and mitotic apparatus. Viruses are quite distinct from the other organisms, as they are not cells but can replicate only within cells.

Characteristics of prokaryotic and eukaryotic cells

Characteristic	Prokaryotic	Eukaryotic
DNA within a nuclear membrane	No	Yes
Mitotic division	No	Yes
DNA associated with histones	No	Yes
Chromosome number	One	More than one
Membrane bound organelles	No	Yes
Size of ribosome	70S	80S
Cell wall containing peptidoglycan	Yes	No

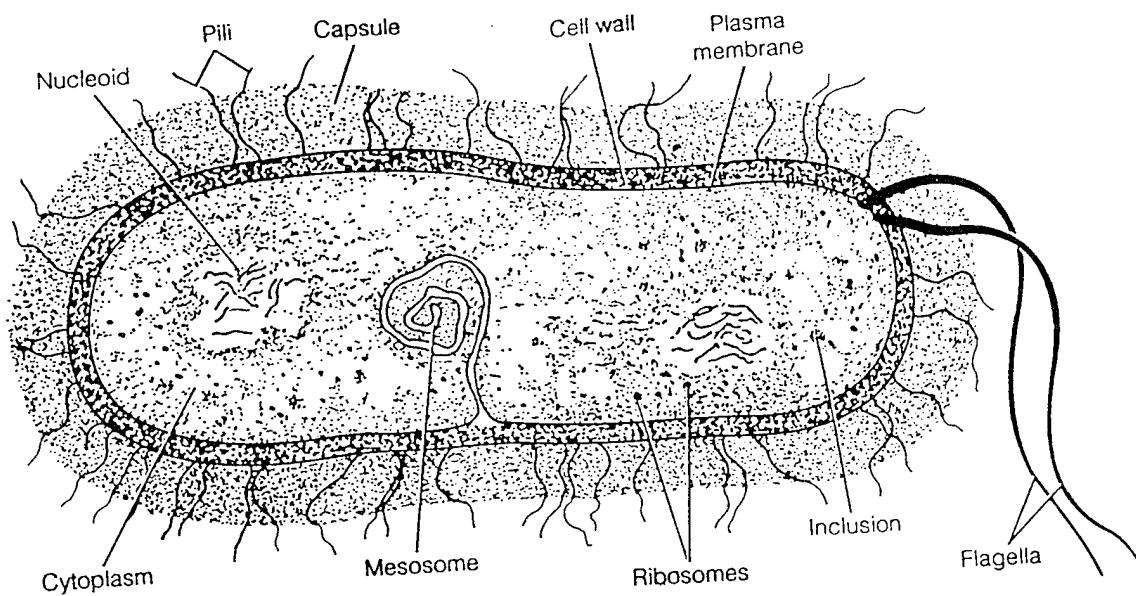
II. Structure of Bacterial Cells

A. Shape and Size: Bacteria have 3 basic shapes (**cocci**, **rods**, and **spirals**) but some are said to be **pleomorphic**



Bacterial morphology. A: Cocci: in clusters, e.g. *Staphylococcus* (A-1); chains, eg, *Streptococcus* (A-2); in pairs with pointed ends, eg. *Streptococcus pneumoniae* (A-3); in pairs, eg, *Neisseria* (A-4). B: Rods: with square ends, eg, *Bacillus* (B-1); with rounded ends, eg, *Salmonella* (B-2); club-shaped, eg. *Corynebacterium* (B-3); fusiform, eg, *Fusobacterium* (B-4); comma-shaped, eg, *Vibrio* (B-5). C: Spirochetes: relaxed coil, eg. *Borrelia* (C-1); tightly coiled, eg, *Treponema* (C-2).

B. Structure: compared to eukaryotic cells bacteria are amorphous forms with little detail



1. Cell Surfaces:

-Capsule: Many bacteria synthesize carbohydrate polymers that form layers on the outside of the cell. Capsules are important for survival and also serve as virulence factors. They are immunogenic, and antigenic differences exist in capsules within a species. *Streptococcus mutans* is an example of oral microflora that produces extracellular polysaccharide capsules. These polymers are important in the accumulation of dental plaque.

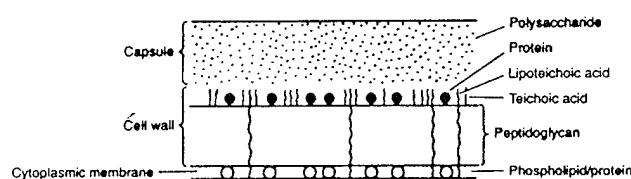
-Flagella: long, threadlike structures that are the organs of motility in bacilli, most vibrio, and spirilla. Such structures are important for chemotaxis and identification.

-Pili: these are fine, hairlike, proteinaceous appendages also known as fimbriae and are found most often on gram-negative bacteria. There are two types. One mediates bacterial adsorption to host tissues (somatic pili) and the other mediate the transfer of genetic material between cells by conjugation and episome transfer (conjugal or sex pili).

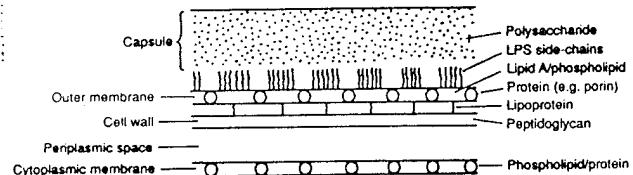
2. Bacterial Cell Envelope:

-Cell Walls: cell walls are present on all bacteria except mycoplasmas. They are located immediately outside the plasma membrane and protect the cell from lysis resulting from osmotic pressure differences or mechanical damage. They also give the cell its characteristic shape, determine its gram stain reaction, and impart antigenic characteristics. Gram-negative cell walls are generally more chemically and structurally complex than are those of gram-positive bacteria. Gram-negative walls (right below) consist of a thin layer of peptidoglycan enclosed by a phospholipid outer membrane containing endotoxin. Gram-positive cell walls (left below) consist of only one peptidoglycan layer that is thicker than seen in the gram-negative cell.

Gram-positive



Gram-negative



-Cytoplasmic Membrane: just inside the peptidoglycan layer of the cell wall lies the cytoplasmic membrane, which is composed of a phospholipid bilayer similar in microscopic appearance to that in eukaryotic cells. They are chemically similar, but eukaryotic membranes contain sterols, whereas prokaryotes do not. The membrane has 5 very important functions:

- active transport of molecules into the cell,
- energy generation by oxidative phosphorylation,
- synthesis of precursors of the cell wall,
- secretion of enzymes and toxins, and
- can control the activity of antibiotics.

-Mesosome: usually seen as cytoplasmic sacs that contain whorled, lamellar, tubular, or vesicular structures and are often associated with division septa

3. Cytoplasm

-Nucleoid: also known as the nuclear body, chromatin body, nuclear equivalent, or nucleus appears as a compact, electron-transparent, centrally located region in the bacterial cytoplasm. Although it performs many of the same functions, the bacterial nucleus varies from nuclei of eukaryotic cells in a number of ways. (1) it is not separated from the rest of the cytoplasm by a nuclear membrane. (2) Discrete chromosomes cannot be observed. (3) There are no histones associated with the DNA. (4) There is no mitotic apparatus.

-Plasmid: extrachromosomal, double-stranded, circular DNA molecules that are capable of replicating independently of the bacterial chromosome. They can, however, also be integrated into the bacterial chromosome. Transmissible plasmids can be transferred from cell to cell by conjugation. Non-transmissible plasmids are small and they are frequently present in many copies per cell. Plasmids can be responsible for coding (1) antibiotic resistance, (2)pili, and (3)exotoxins.

-Ribosomes: dense spherical particles responsible for protein synthesis.

-Inclusion Granules: Most bacteria contain intracytoplasmic polymer granules that are reserves of energy and raw materials stored in a form that will not upset the osmotic balance of the cell. The most common types are metachromatic granules, polysaccharide granules and poly- β -hydroxybutyrate granules.

III. Classification, Nomenclature and Identification

Classification, nomenclature, and identification are the three separate, but interrelated, areas of taxonomy. Classification is the arranging of organisms into taxonomic groups (taxa) on the basis of similarities or relationships. Nomenclature is the assignment of names to the taxonomic groups according to international rules. Identification is the process of determining that a new isolate belongs to one of the established, named taxa. Classification of organisms requires knowledge of their characteristics. For bacteria, this knowledge is obtained by experimental as well as observational techniques, because biochemical, physiological and genetic characteristics are often necessary, in addition to morphological features, for an adequate description of a taxon. The process of classification may be applied to existing, named, taxa or to newly described organisms. If the taxa have already been described, named, and classified, either new characteristics about the organisms or a reinterpretation of existing knowledge of characteristics is used to formulate a new classification. However, if the organisms are new, i.e. cannot be identified as existing taxa, they are named according to the rules of nomenclature and placed in an appropriate position in an existing classification. There is no "official" classification of bacteria. This is in contrast to bacterial nomenclature, where each taxon has one and only one valid name, according to internationally agreed-upon rules, and judicial decisions are rendered in instances of controversy about the validity of a name.

A. Bacterial Taxonomy: the means of classifying microbes

1. **Bacterial species:** the basic unit of classification. A bacterial species is a group of organisms that share a set of characteristics. The concept of a bacterial species is less definitive than for higher organisms. Where morphologic criteria are useful for higher organisms such features alone are usually of little classificatory significance for bacteria since most prokaryotic organisms are too simple morphologically to provide much useful taxonomic information.

Generic name and specific epithet (*Streptococcus mutans*)

2. **Traditionally ordered schemes:** Kingdom Prokaryotae)- not practical for bacteria since there is no fossil record. Classification systems for many higher organisms are based upon evolutionary evidence obtained from the fossil record and appropriate sedimentary dating procedures. Such classifications are termed "natural" or "phylogenetic", and are distinguished from "practical" or "artificial" classifications, which are based

entirely on phenotypic characteristics.

3. Numerical Taxonomy and species definition: Numerical Taxonomy or Cluster Analysis was developed in the late 1950's as part of multivariate analysis and in parallel with the development of computers. Its aim was to devise a consistent set of methods for classification of organisms. Much of the impetus in bacteriology came from the problem of handling the tables of data that result from examination of their physiological, biochemical and other properties. Bacterial taxonomists have agreed that certain ranges of similarity among strains are generally appropriate for inclusion in the same species or genus. With respect to clusters based on phenotypic similarity, strains in the same genus must match at least 65% of the characteristics measured for one or more other strains. Strains in the same species, however, are 70% to 80% similar. With respect to groups based on DNA sequences similarity, strains in the same genus usually form 25% to 60% hybrid molecules that are 8% to 15% divergent. Strains in the same species, called a **genospecies**, form 70% hybrid molecules which are less than 6% divergent.

-Subspecies: A species may be divided into two or more subspecies based on minor but consistent phenotypic variations within the species or on genetically determined clusters of strains within the species. It is the lowest taxonomic rank that has official standing in nomenclature.

Lactobacillus casei subsp. *alactosus*

-Infrasubspecific: Ranks below subspecies, such as those indicated in the table below, are often used to indicate groups of strains that can be distinguished by some special character, such as antigenic makeup, reactions to bacteriophage, or the like. Such ranks have no official standing in nomenclature but often have great practical usefulness.

Infrasubspecific Ranks

Preferred Name	Synonym	Applied to Strains
Biovar	Biotype	Special biochemical or physiological properties
Serovar	Serotype	Distinctive antigenic properties
Pathovar	Pathotype	Pathogenic properties for certain hosts
Phagovar	Phagotype	Ability to be lysed by certain bacteriophages
Morphovar	Morphotype	Special morphological features

3. Genetic Basis For Classification

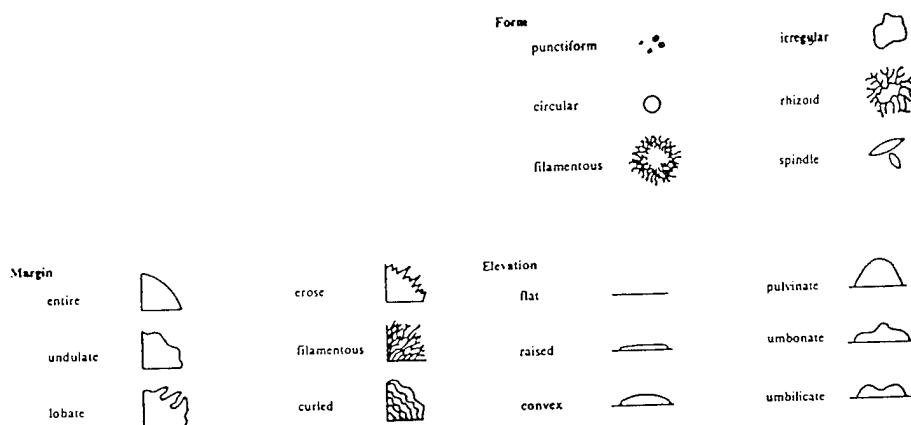
-Nucleic acid homology: based on a comparison of nucleic acid base sequences.

Comment: As new techniques and procedures change, the classification of some bacteria changes. This is common with the oral anaerobic bacteria. For example the bacterium *Porphyromonas gingivalis* has recently been changed from *Bacteroides gingivalis*.

B. Identification

1. **Pure culture:** before any identification is made a pure culture is required. This generally involves plating the organism in such a manner that it becomes increasingly diluted. Once this is done and the bacteria have grown on various media the **colony morphology** can be described. Such morphology is very useful in the identification process.

-Colony morphology: colony morphology can be very indicative of the kind or organism.



2. **Microscopic morphology and staining reactions:** the determination of the gram reaction and the identification of the morphology of the cell are essential to the identification process. Once this is accomplished, the organisms can be placed in one of several large groupings (gram positive cocci, for example) and then can be further identified as indicated below.

3. **Biochemical characteristics:** determination of fermentative properties or ability to utilize or produce some unique compound. For identification of anaerobic oral bacteria it is important to utilize gas-liquid chromatographic techniques.

4. **Serologic reactions:** identification based on the ability of certain organisms to bind specific antibodies.

5. **Bacteriophage typing:** the use of specific bacterial viruses to identify bacterial strains.

6. Pathogenicity: some organisms can be identified, and classified, by their ability to cause disease only in certain species.

7. Antibiotic sensitivity: this is useful for identifying organisms and will indicate the presence of a plasmid. Such sensitivity, however, can be gained or lost.

LECTURE 2: BACTERIAL PHYSIOLOGY AND METABOLISM

I. **Bacterial Growth:** Growth is the coordinated increase of all cellular constituents leading to replication of individual cells and, consequently, to an increase in the cell population. All microbial activities, beneficial or harmful, depend on the ability of the organism to survive in its environment. Growth occurs only when all necessary nutrients are available. Microbes display considerable diversity in use of nutrients. In some instances the tooth can be considered a nutrient.

A. Nutrient Requirements

1. **Carbon:** when the basic requirements of every organism for a source of energy and a source of carbon is considered, microbes fall into four distinct categories:

-**photoautotrophs** derive energy from photosynthesis, and utilize CO₂ as their main source of carbon

-**photoheterotrophs** also use light for energy, but need an organic compound as the principal carbon source

-**chemoautotrophs** derive their energy from chemical reactions involving oxidation of compounds such as H₂, NH₃, or H₂S and utilize CO₂ as their carbon source

-**chemoheterotrophs** derive energy from a chemical source, usually an organic compound, either by oxidizing or fermenting it. In addition, chemoheterotrophs utilize organic compounds as their source of carbon. This group contains all bacteria of interest to the dental community.

3. **Growth factors:** B complex vitamins, amino acids, purines, and pyrimidines

4. **Inorganic ions:** magnesium, potassium, iron, manganese, zinc, copper, cobalt, molybdenum, selenium

5. **Oxygen:** the oxygen requirement of a particular bacterium reflect the mechanism employed for satisfying its energy needs. On the basis of their oxygen requirements, bacteria may be divided into five groups.

-**obligate anaerobes** that grow only under conditions of high reducing intensity and for which oxygen is toxic.

-**aerotolerant anaerobes** that are not killed by exposure to oxygen.

-**facultative anaerobes** that are capable of growth under both aerobic and anaerobic conditions.

-**obligate aerobes** that require oxygen for growth.

-**microaerophilic** organisms that grow best at low oxygen tensions, high tensions being inhibitory.

Anaerobic bacteria have been shown to be prominent among dentally important organisms.

6. Carbon dioxide: The presence of carbon dioxide can also affect bacterial growth. Because CO₂ is directly incorporated into several metabolic pathways, atmospheric levels of this gas are sometimes not enough to allow even heterotrophic organisms to start growing. This requirement can be satisfied by the organism's low metabolism once growth begins, but many bacteria grow better in an atmosphere enriched with CO₂.

B. Physical Requirements

1. Oxygen-reduction potential: a measure of the tendency to give up electrons. E_h is expressed with reference to the hydrogen electrode. Most media range from +200 to +400 mv. Obligate anaerobes grow in media with an E_h of -200 mv.

2. Temperature: Bacteria are divided into three classes based on their optimum growth temperature

Classes Of Bacteria Based On Growth Temperature

Class	Optimum Temperature (°C)	Range (°C)
Psychrophiles	20	0-30
Mesophiles	37	10-45
Thermophiles	50-55	25-90

3. Hydrogen ion concentration: Most bacteria grow at a neutral or slightly alkaline pH, although many will tolerate a pH between 5 and 9. Intracellular pH is maintained close to neutrality. Internal neutrality is necessary to protect constituents such as nucleic acids and high energy compounds that are susceptible to either acid or alkali. Environmental pH is one condition that is altered readily by bacterial, metabolic activity, with consequences that can be beneficial or detrimental to either the bacteria or the host. Demineralization of tooth enamel at pH 5.5 and below would be an example of a detrimental consequence.

4. Osmotic conditions: bacteria are generally osmotically tolerant.

C. Uptake Of Nutrients

1. Extracellular enzymes: aid in uptake of nutrients by breaking down food materials.

2. Membrane transport: can occur as a result of the following:

-passive transport: occurs only if there is a concentration gradient across the membrane.

-facilitated diffusion: a mechanism by which substances are transported from an area of high concentration to one of relatively low concentration without energy expenditure.

Facilitated diffusion is specific because it is mediated by membrane-bound proteins, called **permeases**, that bind molecules on the outer surface, carry them through the membrane, and release them unchanged into the cytoplasm. It is faster than passive diffusion.

-**active transport**: a mechanism that allows solutes to enter the cell against a concentration gradient via specific membrane-bound permeases. This is an energy requiring mechanism.

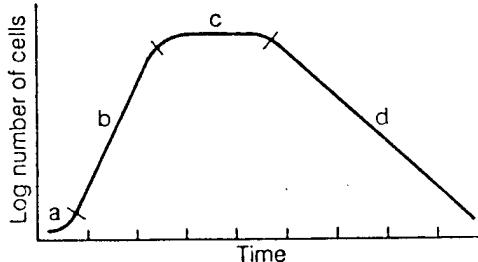
-**group translocation**: a transport mechanism in which the substrate is modified chemically on its way into the cell to a form that cannot pass back out of the cytoplasm. It requires energy expenditure, but unlike active transport, there is very little increase in the cytoplasmic concentration of unmodified substrate.

D. Growth Cycle: In vitro bacterial growth can be studied using batch cultures, continuous cultures, or synchronous growth techniques in which all cells in a culture are maintained in the same phase of the cell cycle. Growth measurement in batch culture results in a four phase curve.

1. **Lag phase**: a period of adjustment in which there is intense physiologic activity as bacteria inoculated into the medium adapt to their new environment. The population size remains constant during this time.

2. **Exponential growth phase**: cells are dividing steadily, and the population increases at a constant rate, called the generation time. During the exponential growth phase or log phase, the growth rate is maximal, and the population is most nearly uniform in chemical composition and metabolic activity.

3. **Stationary phase**: since batch cultures are closed systems, an exponential culture soon will change its own environment by depleting essential nutrients and by producing end products that adversely affect the cells. The effect of this change is a slower growth rate, reflected in the stationary phase. During this time the, the population remains constant due to a balance between the number of cells dividing and those dying.



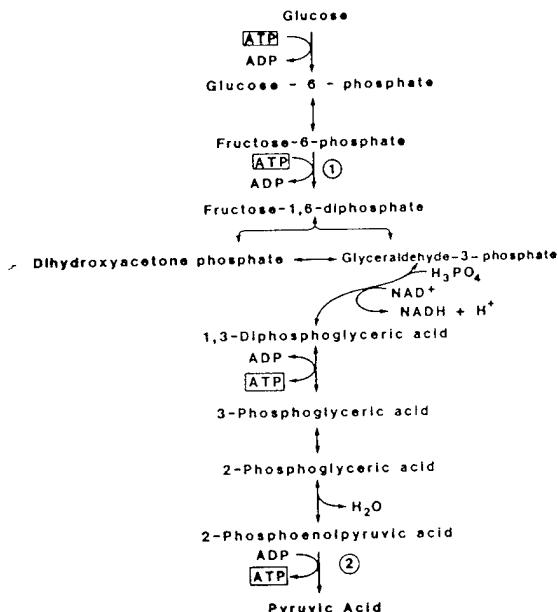
4. **Phase of decline**: the final phase, also known as the **death phase**, reflects an increasing number of cells dying relative to living, multiplying cells.

II. Energy Metabolism: The sum total of all chemical reactions which occur in the cell is termed **metabolism**. Much of the metabolic effort of bacteria is devoted to the stepwise degradation of substrates so that their intrinsic bond energy is conserved in a biologically useful form.

A. Energy-Yielding Heterotrophic Metabolism: Although all heterotrophic microorganisms ultimately obtain their energy from oxidation-reduction reactions, the amount of energy obtained and the mechanisms by which they extract it vary. During fermentation, electrons are passed from the electron donor to an electron acceptor, which is some other organic intermediate in the fermentation process. Fermentation results in the accumulation of a mixture of end products. Fermentations

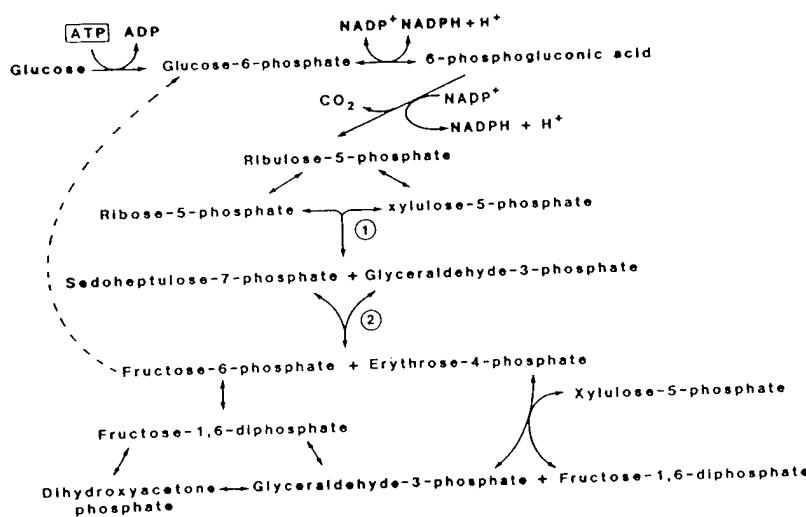
are carried out by obligate and facultative anaerobes. Respiration is a process in which molecular oxygen usually serves as the ultimate electron acceptor. When oxygen is the ultimate acceptor the process is referred to as **aerobic respiration**. When an inorganic compound, such as nitrate, sulfate, or carbonate, is used the process is referred to as **anaerobic respiration**.

1. Glycolytic Pathway:

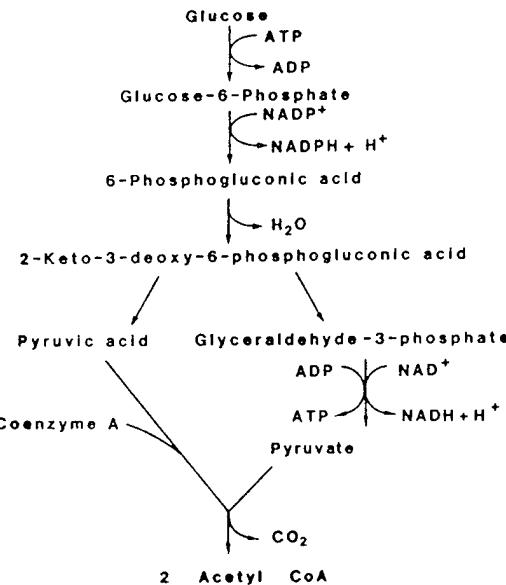


when an organic compound such as glucose enters the cell it will be degraded by a series of reactions-one such series is the Embden-Meyerhof-Parnas glycolytic pathway. Under aerobic conditions the hydrogen from NADH is transferred to the electron transport system and ultimately to oxygen. Under anaerobic conditions, pyruvate or some other organic compound can be reduced and act as the hydrogen acceptor.

2. Phosphogluconate Pathway (pentose shunt): a mechanism for catabolism of hexoses



3. Entner-Doudoroff Pathway: one of a number of other systems found in bacteria.



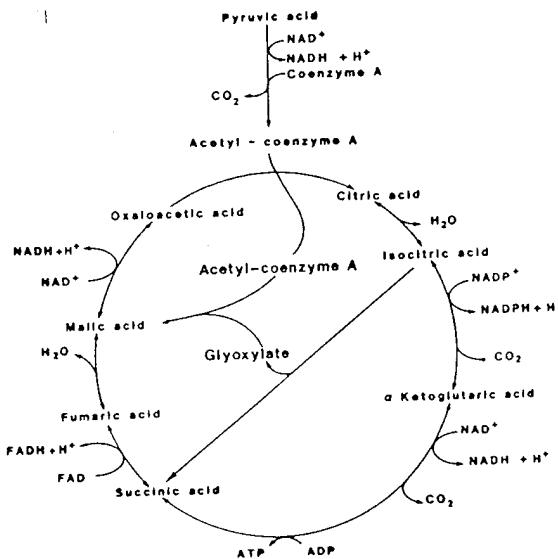
B. Fate of Pyruvate under Anaerobic Conditions

1. **Alcoholic Fermentation:** oldest known system
2. **Homolactic Fermentation:** high yield of lactic acid
3. **Heterolactic Fermentation:** Lactobacillus
4. **Propionic Acid Fermentation:** Propionibacterium (Swiss cheese)
5. **Mixed Acid Fermentation:**
6. **Butanediol Fermentation**
7. **Butyric Acid Fermentation**

8. Fermentation of Nitrogenous Organic Compounds

C. **Aerobic Respiration:** In aerobic cells, energy is obtained from the complete oxidation of the substrate, with molecular oxygen usually serving as the ultimate hydrogen acceptor. In respiration the large amount of energy set free in the formation of water is made available to the process. The pathways of aerobic dissimilation are exceedingly complex. They consist of many enzymes and a large number of biochemical reactions. The most important respiratory mechanism for terminal oxidation is the tricarboxylic acid cycle of Krebs, which, together with the known reactions of glycolysis, can account for the complete oxidation of glucose. This cycle is unique in that it provides the cell not only with an energy source but also with carbon skeletons for the synthesis of cellular material.

1. Tricarboxylic Acid Cycle: The Krebs tricarboxylic acid cycle, and within it the "glyoxylate bypass" is shown below. The circled compounds represent sources of biosynthetic drain, and in an organism growing on acetate alone the glyoxylate cycle provides net 4 carbon synthesis to the extent required to replenish this drain.



2. Electron transport system: the electron transport system carries out the final stage of respiration, in which the substrate is fully oxidized and the electrons released are transferred to a terminal acceptor, most commonly oxygen.

III. Anaerobic Bacteria: the anaerobic bacteria are widespread in nature. They constitute the predominate part of our normal indigenous flora on mucocutaneous surfaces and outnumber facultatively anaerobic bacteria in the gut by a factor of 1000:1. In the mouth, and on the skin, upper respiratory tract, and female genitourinary tracts, they outnumber facultatively anaerobic bacteria by a factor of 5 to 10:1. Many of these anaerobic organisms previously considered to be harmless commensals of our indigenous flora, are now recognized as opportunistic pathogens that may produce disease when the host's resistance is reduced.

A. Types of Anaerobic Organisms: -the anaerobic bacteria include a variety of morphologic types, including bacilli, cocci, comma-shaped organisms, and spirochetes. As a group they also tend to be more pleomorphic than most aerobic or facultative organisms.

B. Physiology

1. Anaerobiosis-complete understanding of oxygen intolerance among anaerobic bacteria remains unresolved. Various factors play a role, but no single mechanism has yet been accepted. Some proposals include:

- a. O₂ has a direct toxic effect.
- b. O₂ is indirectly toxic via specific mediators, such as H₂O₂ or free radicals.
- c. An appropriately low oxidation-reduction potential that appears to be required for many anaerobic bacteria is not achievable in the presence of normal O₂ tensions.
- d. Essential sulfhydryl-containing enzymes are oxidized and therefore inactivated by O₂.
- e. O₂ inhibits metabolism by reaction with flavoproteins and reduced nicotinamide adenine dinucleotide (NADH) oxidases, thereby critically lowering the reducing power of the cell.
- f. Anaerobes lack enzymes such as catalase (or peroxidase) and superoxide dismutase, which thereby allows accumulation of toxic levels of H₂O₂ or superoxide ions, respectively.

C. Culture and Identification

1. **Culture Systems:** Growth requirements are met by addition of yeast extract, blood, serum, ascites, vitamin K, hemin, and a fermentable carbohydrate.

2. **Isolation:** must be done in the absence of oxygen

- a. Jar technique:
- b. Prereduced anaerobically sterilized media (PRAS)
- c. Roll-tube procedures

3. Identification

a. **Selective media:** specimens should be cultured as soon as possible. Many infections are polymicrobial and the nutritive material present will support growth of the least fastidious organisms.

b. **Gas-liquid chromatography (GLC):** Used to identify the volatile fatty acids, non-volatile fatty acids, and alcohols that are indicative of certain anaerobes.

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VIRAL INFECTIONS OF DENTAL IMPORTANCE
August 31, 1995, 0730 - 0930

I. GENERAL PROPERTIES OF VIRUSES

A. Basic Terminology

1. Definition of a Virus
2. Chemical Composition of Viruses
 - a. Nucleic acid -
 - b. Proteins -
 - c. Lipid envelope -
3. Morphology
 - a. Size -
 - b. Capsid -
 - c. Nucleocapsid -
 - d. Envelope -
 - e. Symmetry -
4. Contrasting Viral Properties with other Microorganisms:

<u>Microorganisms</u>	<u>Viruses</u>
DNA & RNA	DNA or RNA
Ribosomes	--
Binary Fission	--
Not all are parasitic	Obligate parasite
Insensitive to interferon	Sensitive to interferon
Sensitive to antibiotics	Insensitive to antibiotics

5. Classification based on

- a. Structure (see Table 32-1 and Figure 32-4)
- b. Symptoms

B. Viral Replication

► See chapter on 'Multiplication of Viruses: An Overview' by Bernard Roizman,
Virology (B.N. Fields), 1985.

1. Attachment and Penetration
2. Uncoating
3. Replication
 - a. DNA viruses
 - b. RNA viruses
 - i. positive sense
 - ii. negative sense
 - iii. retroviruses
4. Assembly, Maturation and Release
5. One-step growth curve

C. Virus-Cell Interactions

1. Lytic infection (cytoidal) -
2. Persistent, productive infection (chronic infection) -
3. Latent infection -
4. Slow infections -

II. VIRUSES OF DENTAL IMPORTANCE

A. Hepatitis causing viruses

1. HAV - Picornavirus

2. HBV - Hepadenavirus

3. HCV - Flavivirus-like

4. HDV - Co-infection with HBV (a sneaky RNA virus in HBsAg coat!)

5. HEV - Calicivirus

B. Retroviridae

▷ Enveloped RNA viruses that make a DNA provirus that integrates into the host cell's DNA and stays there forever. Three types: Oncovirus, lentivirus, spumavirus.

1. HTLV-I

2. HIV

C. Herpesviridae

▷ Seven human herpes viruses cause a variety of clinical diseases. A hallmark of herpesvirus infection is their ability to produce latent infections.

1. HSV (types 1 and 2)

2. VZV - Chickenpox and Shingles

3. EBV - Mononucleosis

4. CMV - Immunocompromised hosts

5. HHV-6

D. Orthomyxoviridae

1. Influenza virus

E. Paramyxoviridae

1. Parainfluenzavirus

2. Mumps

3. Measles

4. Respiratory syncytial virus

F. Rubiviridae

1. Rubella virus

G. Picornaviridae

1. Rhinovirus

2. Enterovirus

- Polio -
- Coxsackie -

H. Papovaviridae

1. Papillomavirus

I. Adenoviridae

1. Adenovirus

III. PREVENTION AND TREATMENT

A. Protective measures

B. Vaccinations

1. Ideal vaccines

2. Killed versus Live vaccines

3. Future prospects

a. Genetically engineered vaccines

b. DNA vaccines

C. Drugs (see handout on anti-viral drugs)

Table 32-1. Classification of viruses into families based on chemical and physical properties.

Nucleic Acid Core	Capsid Symmetry	Virion: Enveloped or Naked	Ether Sensitivity	No. of Capsomers	Viral Particle Size (nm) ¹	Weight of Nucleic Acid in Virion ($\times 10^6$)	Molecular Weight of Nucleic Acid	Physical Type of Nucleic Acid	No. of Genes (approx.)	Viral Family
DNA	Icosahedral	Naked	Resistant	32	18–26	1.5–2.2	ss	ds circular ds	3–4	Parvoviridae
				72	45–55	3–5	5–8		Papovaviridae	
				252	70–90	20–30	30		Adenoviridae	
	Complex	Enveloped	Sensitive	162	100 ²	90–130	ds		160	Herpesviridae
			Resistant ³		230 × 400	130–200	ds		300	Poxviridae
		Complex coats			42	1.6	ds circular ⁴		4	Hepadnaviridae
RNA	Icosahedral	Naked	Resistant	32	20–30	2.3–2.8	ss	ss ds segmented	4–6	Picornaviridae
				32 ⁵	35–39	2.6	4–6		Caliciviridae	
					60–80	12–15	10–12		Reoviridae	
	Unknown or complex	Enveloped	Sensitive	327	50–70	4	ss		10	Togaviridae
			Sensitive		45–50	4	ss		10	Flaviviridae
					50–300 80–160 ~100	3–5 7 7–10	ss segmented ss diploid		10 30 4	Arenaviridae Coronaviridae Retroviridae
Helical	Enveloped		Sensitive		90–100	6–15	ss segmented	>3 10 >10 5	Bunyaviridae Orthomyxoviridae Paramyxoviridae Rhabdoviridae	
					80–120 150–300	5 5–7 4	ss segmented ss ss			

¹Diameter, or diameter \times length.²The naked virus, ie, the nucleocapsid, is 100 nm in diameter; however, the enveloped virion varies up to 200 nm.³The genus *Orthopoxvirus*, which includes the better studied poxviruses (eg, vaccinia, variola, cowpox, ectromelia, rabbitpox, monkeypox), is ether-resistant. Some of the poxviruses belonging to other genera are ether-sensitive.⁴One strand has a constant length of 3182 bases, and the other varies between 1700 and 2800 bases.⁵Reoviruses possess a double protein capsid shell in which the exact number and spatial arrangement of capsomers are difficult to determine.
ss = single-stranded
ds = double-stranded

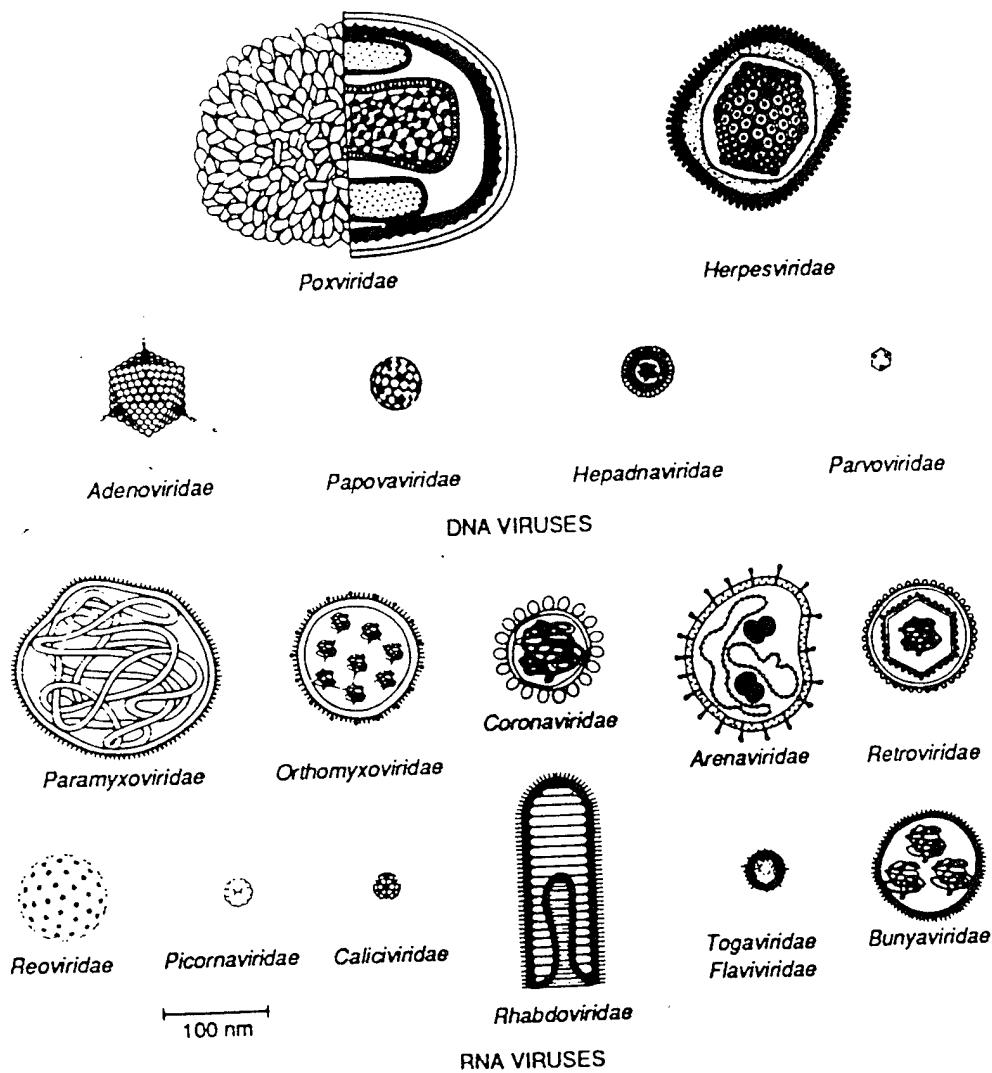


Figure 32–4. Shapes and relative sizes of animal viruses of families that include human pathogens. In some diagrams, certain internal structures of the particles are represented. (Reproduced, with permission, from White DO, Fenner FJ: *Medical Virology*, 3rd ed. Academic Press, 1986.)

Lecture 3: ORAL MICROBIAL ECOLOGY

The understanding that dental caries and periodontitis are caused by members of the commensal oral microflora has stimulated considerable interest in attempting to identify the specific microorganisms associated with these disease states. However, this has directed attention away from studies of the oral flora as a whole and had focussed attention toward gram-positive cocci and bacilli.

Several hundred different bacteria, fungi, and protozoans can live in the oral cavity. When these organisms adhere to some surface they form an organized mass called **dental plaque**. Our understanding of how these organisms arrive in the plaque community and how they interact with each other and their hosts has been improved immeasurable by applying the principles of ecology to the study of this complex system. At this time you will be given an overall view of the oral cavity and the oral microbiota from the point of view of microbial ecology. Therefore, many of the concepts that will be covered in more detail later will be introduced here.

I. Oral Ecology-the study of the interrelationships between living things and their environment. The term **environment** in this context means the total of the physical, chemical, and biological characteristics of the surroundings (including other living organisms) that are potentially available for habitation. The word **environment** can be used to describe an organism's surrounding at a number of different levels of complexity (all inclusive environment, macroenvironment, microenvironment). In contrast, a **niche** is the specific combination of physical, chemical, and biological parameters that are necessary for the survival of a particular organism. The interaction between the commensal normal flora and its host is considered an **ecosystem**. As in all ecosystems, this relationship is often fragile and can be disturbed by minor shifts in the balance of a variety of factors.

A. The Oral Ecosystems-the bacteria that normally reside in the oral cavity (**indigenous** flora as opposed to **transient** bacteria, which are unable to become established in the oral cavity) are distributed in different proportions depending on where in the oral cavity one looks. The oral cavity can be divided into major ecosystems on the basis of the distribution of the indigenous flora and by means of physical and morphological criteria.

1. buccal epithelium:
2. dorsum of the tongue:
3. supragingival tooth surface:
4. subgingival tooth and crevicular epithelial surfaces:

B. Ecological Determinants-there are a variety of factors in the oral cavity which predispose toward the selection of certain microbes. The unique topographical arrangements, varied terrain, presence of teeth, crevices, lips, and the tongue represent obvious factors. Other factors are:

1. Physicochemical Factors

a. **Hydrogen ion concentration:** the term pH refers to the negative logarithm of hydrogen ion concentration; thus, the lower the pH value, the higher is the hydrogen ion concentration. The pH in the oral cavity is fairly constant (6.8-7.2). This is generally a result of the buffering capacity of saliva and gingival fluid as well as mechanical sloshing of saliva into less accessible areas. A low pH at a particular site in the mouth due to production of acid by bacteria metabolizing carbohydrates would deter the development of nonaciduric microorganisms. A low pH is due to the presence of large numbers of acidogenic microorganisms, such as lactobacilli and certain streptococci, which degrade fermentable carbohydrates to acid.

b. **Temperature:** mesophilic organisms predominate. Bacteria tend to be more fastidious in temperature requirements and therefore the changes in temperature may cause significant changes in flora.

c. **Oxygen tension (Eh):** The presence of natural teeth or dentures favors an anaerobic flora. This is due to a buildup of microflora that use oxygen, resulting in a lowering of the oxygen-reduction potential. Outer layers of bacteria in dental plaque tend to be aerobic and use oxygen, permitting anaerobic bacteria to proliferate in the deeper layers. The environment in the gingival sulcus is different from that of other oral ecological niches. The Eh is low, allowing the growth of anaerobic species.

d. **Adherence and Aggregation:** Oral bacteria can bind uniquely to the surfaces in the oral cavity. *S.sanguis* and *S.mutans* bind to tooth surfaces. While *S.salivarius* do not bind to teeth they do bind well to epithelial cells. The tongue is colonized by *Veillonella*.

e. **Availability of Nutrients:** when the nutrient requirements of the oral microbiota are considered, the oral cavity can be thought of as consisting of two major environmental systems. Organisms in supragingival plaque and those which inhabit mucosal surfaces are bathed primarily in saliva, while organisms that inhabit the subgingival crevice or periodontal pocket are bathed primarily in crevicular fluid. Crevicular fluid is an inflammatory exudate derived from plasma. Therefore, we can consider these two environments as one that is bathed in saliva (supragingival) or as one that is bathed in plasma (subgingival). Two main categories of nutrients in saliva are carbohydrates and amino acids. Crevicular fluid since it is obtained from plasma is an excellent source of nutrients.

2. Host Factors: Saliva

a. **Antibodies:** secretory immunoglobulin A

b. **Glycoproteins:** a large number of glycoproteins are present in saliva

c. **Nonspecific Factors:** factors that specifically inhibit bacteria; lysozyme, lactoferrin, lactoperoxidase

3. Host Factors: Crevicular fluid

a. **Antibodies:** predominant is immunoglobulin G

b. **Complement:**

4. Bacterial determinants: the bacteria that exist in the oral cavity must be able to use the nutrients available and to survive under the physical and chemical conditions present therein. Likewise, they must have some degree of resistance to the host defense mechanisms.

a. **Adherence:** one of the major ways in which bacteria "choose" the appropriate milieu to colonize is by **selective adhesion**.

b. **Interaction with other microbes:** certain bacteria apparently allow other bacteria to colonize subgingival areas more easily. Some bacteria are able to use nutrients and other substances made by other organisms.

5. Factors Under Host Control: two major factors that are under the conscious control of human beings greatly influence the composition of oral flora.

a. **Dietary habits:**

b. **Oral hygiene habits:**

II. Development of the Oral Microflora As A Specific Example of Ecological Succession

At birth, the oral cavity is sterile and only low numbers of microorganisms can be recovered during the first 6 hours. From 6 hours to 10 hours after birth, the number of organisms appears to increase rapidly. Species of *Streptococcus*, *Staphylococcus*, *Veillonella*, and *Neisseria* are generally present in most infants by 12 months. *Actinomyces*, *Lactobacillus*, *Rothia*, and *Fusobacterium* species can be cultivated from more than half and *Bacteroides*, *Candida*, *Leptotrichia*, *Corynebacterium*, and coliforms can be isolated from less than half of infants at one year of age.

EXAMPLES OF SPECIFIC ATTACHMENTS OF MICROORGANISMS

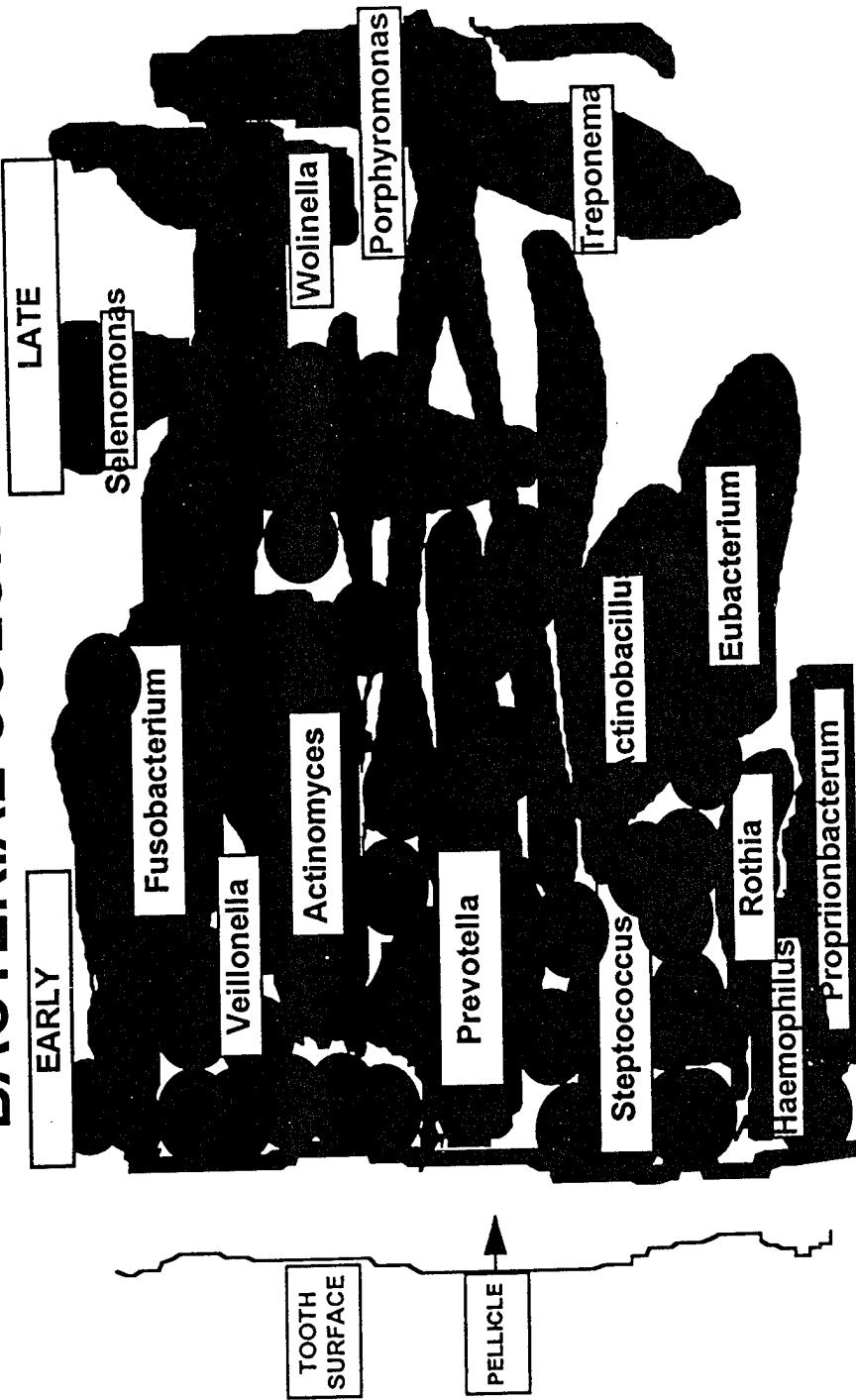
Microorganism	Disease	Attachment Site
Influenza virus	Influenza	Respiratory epithelial cells
Chlamydia	Conjunctivitis, Urethritis	Conjunctival or urethral epithelium
Mycoplasma pneumonia	Atypical pneumonia	Respiratory epithelial cell
Neisseria meningitidis	Carrier state	Nasopharyngeal epithelium
Vibrio cholerae	Cholera	Intestinal epithelium
Salmonella typhi	Enteric fever	Intestinal epithelium
Treponema pallidum	Syphilis	Mucopolysaccharide on cell surface or in tissue
Plasmodium vivax	Malaria	Erythrocyte of susceptible human
Streptococcus mutans	Caries	Tooth
Streptococcus salivarius		Buccal and pharyngeal epithelium
Streptococcus sanguis		Pellicle

A. Microbial Succession: the replacement of one type of microbial community by another in response to modification in the habitat.

1. Autogenic succession: microbial population changes caused by microbes themselves. Results from microbial induced changes in nutrients, pH, E_h , vitamins, supplements. For example, Veillonella cannot ferment and therefore require breakdown products from other organisms. *S.mutans* require p-aminobenzoic acid from *S.sanguis* in plaque.

2. Allogenic Succession: refers to microbial alterations brought about by environmental conditions that are not of microbial origin. Changes in nutrient sources, alterations as a result of immune responses and/or drug treatments.

BACTERIAL COLONIZATION

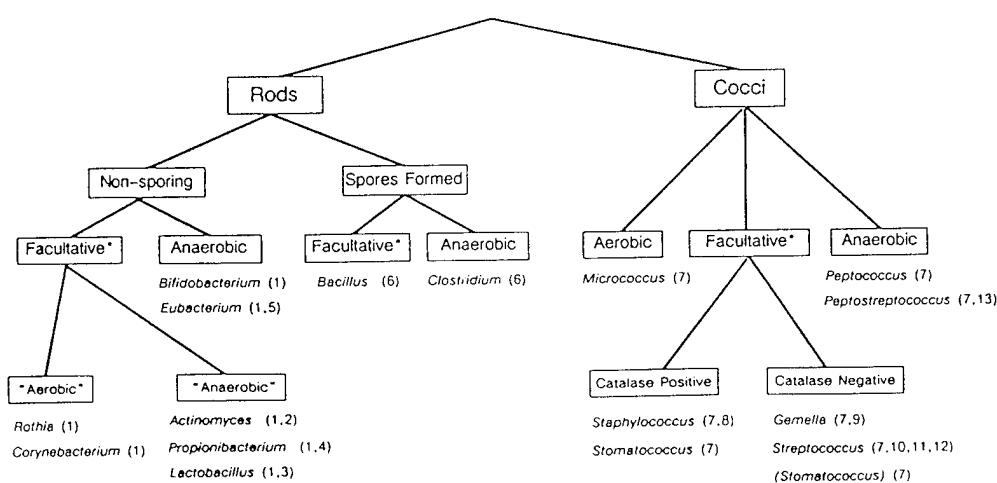


Lecture 4: ENDOGENOUS BACTERIAL FLORA

Nature Of The Oral Microflora: In spite of the difficulties in sampling, enumerating and identifying oral microorganisms, a general picture of the composition of the normal flora of the dentulous mouth can be discerned. Regardless of variations from person to person and from site to site, facultative and anaerobic streptococci, *veillonellae*, and pleomorphic gram positive rods usually comprise up to about 80% of the total viable count. Facultative streptococci plus *veillonellae* constitute the major components of the salivary flora, and this appears to represent mostly those organisms which colonize the dorsum of the tongue. In plaque and gingival sulcus, the proportions of gram positive rods (mainly *Actinomyces* species) and gram negative anaerobic rods (*bacteroides*, *fusobacteria*, *vibrios*) are higher. *Neisseriae* are regularly present (3-5% of the count). *Lactobacilli*, staphylococci and filamentous forms usually each add up to 1% or less of the total. Black-pigmented *Bacteroides* species, spirochetes and some other anaerobic species appear to be indigenous to the gingival sulcus. Yeasts and coliforms are indigenous to about half of adult mouths but occur in small numbers. Mycoplasmas can be demonstrated in all adult mouths. Protozoa can be demonstrated in small numbers in nearly half of clean and healthy adult mouths; their presence in abundance may signify bad oral hygiene.

I. Gram-Positive Bacteria: Many different gram-positive species inhabit the human oral cavity, occurring in dental plaque around teeth and colonizing the mucosal surfaces. In some of these gram-positive organisms the Gram reaction can be variable, weak, or sometimes negative. Gram-positive species range in morphology from cocci to regular and irregular rods. Most important among the cocci are the *Streptococcus* species. A few rod genera such as *Lactobacillus* species have regular cells; but most of the gram-positive rods have characteristically irregular cell morphologies in which the cells have an uneven diameter with swollen ends and are often described as "club-shaped" or "diphtheroid". The cells are often curved or bent, and many species form branching filaments. The major genera, demonstrating this cell morphology include *Actinomyces*, *Propionibacterium*, and *Eubacterium*. The aerotolerance of oral gram-positive bacteria ranges from aerobes to strict anaerobic. Metabolically most of the species are fermentative, producing a range of acid end products from carbohydrates and amino acids. All the major genera of oral gram-positive bacteria include representatives that are involved in extraoral infections, including endocarditis.

The following figure represents a determinative key to the genera of oral gram-positive bacteria:



A. Gram Positive Cocci

1. **Streptococci:** facultative streptococci form the most numerous single group in the oral cavity. The most abundant of the oral streptococci are those referred to collectively as the *viridans* group. Some of the most important streptococci include *S.sanguis* which are predominant in plaque; *S.salivarius* which make up half of the streptococci count from saliva; and *S.mutans* which is responsible for tooth caries. Not all streptococci isolated from the oral cavity are easy to identify.

The streptococci are a heterogeneous group of bacteria that derive their name from their spherical cellular morphology and their tendency to form chains. Streptococci are widely distributed in nature and may be saprophytic and parasitic. They are responsible for a number of diseases in humans and other animals. In humans, these diseases include the respiratory diseases produced by *Streptococcus pyogenes* and *Streptococcus pneumoniae*, dental caries by *Streptococcus mutans*, orofacial infections, and infective endocarditis. Since the streptococci are so varied, it is not possible to describe them as a single group.

2. **Classification Of Streptococci:** the streptococci are differentiated on the basis of immunologic, cultural, and biochemical properties. Many of the streptococci may be immunologically differentiated according to the Lancefield classification. These streptococci contain an acid-soluble carbohydrate antigen (the C carbohydrate) in their cell walls. Nineteen groups have now been identified. Members of the Lancefield groups also possess additional distinctive cell wall antigens that allow further classification. The M protein found in group A is one of the most important. M proteins have been used to subdivide group A into over 60 serotypes. Serotyping is useful in epidemiologic studies and in predicting the potential for sequelae of acute streptococcal infections.

Distinguishing Properties Of Selected Streptococcal Groups

Category	Species	Hemolysis	Feature	Diseases
Group A	S. pyogenes	β	Bacitracin	pharyngitis, scarlet fever, skin infections, rheumatic fever, nephritis
Group B	S. agalactiae	β	CAMP	Neonatal infections
Group D				
Enterococci	S. faecalis	α, β, γ	Growth in salt and at 45°C	Infective endocarditis, urinary tract
Nonenterococci	S. bovis	α, γ	Growth at 45°C	Infective endocarditis
Viridans	S. mitis, S. milleri, S. mutans	α, γ, β		Infective endocarditis
Pneumococcus	S. pneumoniae	α	Optochin and bile	Pneumonia, meningitis, otitis media
Peptostreptococcus	P. magnus	Variable	Anaerobic	Mixed anaerobic infections

a. **Group A Streptococci:** most human pathogens and the agents for the most severe infections are found in group A.

- **Morphology:** gram-positive, spherical organisms that usually divide in one plane and have a tendency to remain attached and to form chains. None of the streptococci are motile, and none form spores. *S.pyogenes* possesses fimbriae or pili. It also produces a hyaluronic acid capsule early in culture that is rapidly lost as the culture grows older.

- **Cultivation:** facultative anaerobes whose growth is optimal at 37° C.

- **Virulence factors:** group A streptococci produce many possible virulence factors, but the actual roles of many of these factors in disease processes are uncertain. The M protein appears to be the most significant factor in disease. It is antiphagocytic and also has been shown to be an adherence factor.

- **Diseases:** see table

b. Group B Streptococci: members of the normal flora in the pharynx, gastrointestinal tract, and vagina. In the past, these organisms were not common causes of serious disease, but group B infections have been increasing in recent years. Give a positive response to the CAMP test.

c. Group D Streptococci: members of the normal flora of the gastrointestinal, respiratory, and genitourinary tracts and skin. They differ from other streptococci because they can grow at higher temperature and in the presence of bile. Group D streptococci are divided into two groups, **enterococci** and **nonenterococci**, based on the ability of the enterococci to grow in the presence of 6.5% salt.

d. Viridans Streptococci: a large number of streptococci that do not fit readily into any of the established classification schemes have been relegated to a large heterogeneous group called the **viridans** or α -streptococci. Despite these names, many are nonhemolytic, and some are β -hemolytic. The viridans streptococci are members of the normal flora of the mucous membranes of the body, including the oral cavity, the nasopharynx, and the genitourinary tract. The viridans streptococci are important because:

- they comprise a major portion of the oral flora of humans and are involved in several oral diseases including caries, and
- they account for most of the cases of subacute infective endocarditis.

e. Peptostreptococcus: a genus of anaerobic streptococci that is found in mixed anaerobic infections in the pelvis, abdomen, lungs, and oral cavity. They have been isolated from polymicrobial anaerobic lesions in the periodontium, the pulp, and other orofacial infections.

f. Streptococcus Pneumoniae: a gram positive, lancet-shaped coccus that usually occurs in pairs but can be found in chains. Virulent obligately anaerobic streptococci are also prevalent. Gram positive cocci of the genus *Peptostreptococcus* have been estimated to average from 4 to 13% of the viable count in different sites in the oral cavity. *P.anaerobius* and *P.micros* are prevalent in infected root canals. Considerable confusion exists concerning the anaerobic cocci and there is clearly a need for more detailed studies.

3. Staphylococci and Micrococci: nearly every mouth harbors facultative, catalase positive, gram positive cocci that ferment glucose and nitrate. They average about 2% of the viable count from the gingival sulcus and up to 6.5% from the dorsum of the tongue. Pyogenic staphylococci are not common in the non-diseased mouth.

C. Gram Positive Rod-Shaped Bacteria: large numbers of gram positive rod-shaped or filamentous bacterial are found in the oral cavity, particularly in supragingival plaque. These include aerobic, facultative and obligately anaerobic species.

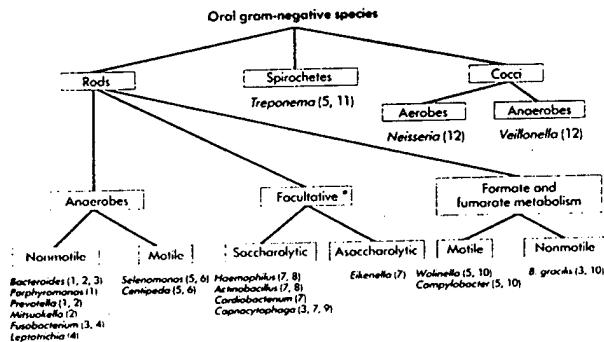
1. **Lactobacilli:** a characteristic group of oral bacteria, although numerically a minor fraction. The number varies with the extent of caries.

D. Pleomorphic Gram Positive Rods and Filaments: Some of the common genera include *Actinomyces*, *Nocardia*, *Rothia*, *Corynebacterium*, *Bacterionema*, and *Leptotrichia*. These organisms tend to be lumped together because of their unusual morphology which include diphtheroids, coryneforms, filaments, coccobacillary forms, and filamentous branching forms.

1. **Actinomyces:** *A.naeslundii* and *A.viscosus* are prevalent in gingival crevice and plaque.

II. Gram Negative Bacteria: the majority of gram-negative species from the gingival sulcus belong to the family of gram negative anaerobic rods, *Bacteroidaceae*. This family includes nonmotile genera including *Bacteroides*, *Porphyromonas*, *Prevotella*, *Mitsuokella*, *Fusobacterium*, and the filamentous *Leptotrichia*. Oral motile, non-spirochetal anaerobes that ferment carbohydrates (saccharolytic) belong in *Selenomonas* and the genetically closely related *Centipeda periodontii*. Rod-shaped species in genera with less fastidious anaerobic requirements coexist with the anaerobes. Genera of facultative saccharolytic rods include *Haemophilus*, *Actinobacillus*, *Cardiobacterium*, and *Capnocytophaga*. *Eikenella corrodens* is a facultative but does not ferment carbohydrates (asaccharolytic). Other asaccharolytic rod species are stimulated to grow by the addition of formate and fumarate to media. These include the motile genera of *Wolinella* and *Campylobacter*, and nonmotile *Bacteroides gracilis*. The highly motile oral spirochetes are strict anaerobes and belong in a single genus, *Treponema*, of the family *Spirochaetaceae*. Aerobic gram-negative cocci belong in *Neisseria*, and anaerobic gram-negative cocci are included in the genus *Veillonella*.

The following diagram is a determinative key to the genera of oral gram-negative species. Such a determinative key is constructed principally from relatively easily measured tests, including Gram stain, cell morphology and motility, aerotolerance, and ability to ferment carbohydrates.



A. Gram Negative Anaerobic Bacteria

1. **Bacteroides:** *B.melaninogenicus* is routinely found in the gingival sulcus and periodontal pocket. The naming of many of these organisms is currently in flux.

2. **Fusobacteria:** Shown to be associated with ulcerative gingivitis. Their classification is also continuing to change. Members of this genus are obligately anaerobic, non-sporeforming rods. Considerable impetus was given to the role of fusiform bacteria in the etiology of oral gangrenous lesions when they were associated with spirochetes in acute ulcerative gingivitis. The fusobacteria and spirochetes greatly increase in inflammatory periodontal disease and decrease with the abatement of the disease.

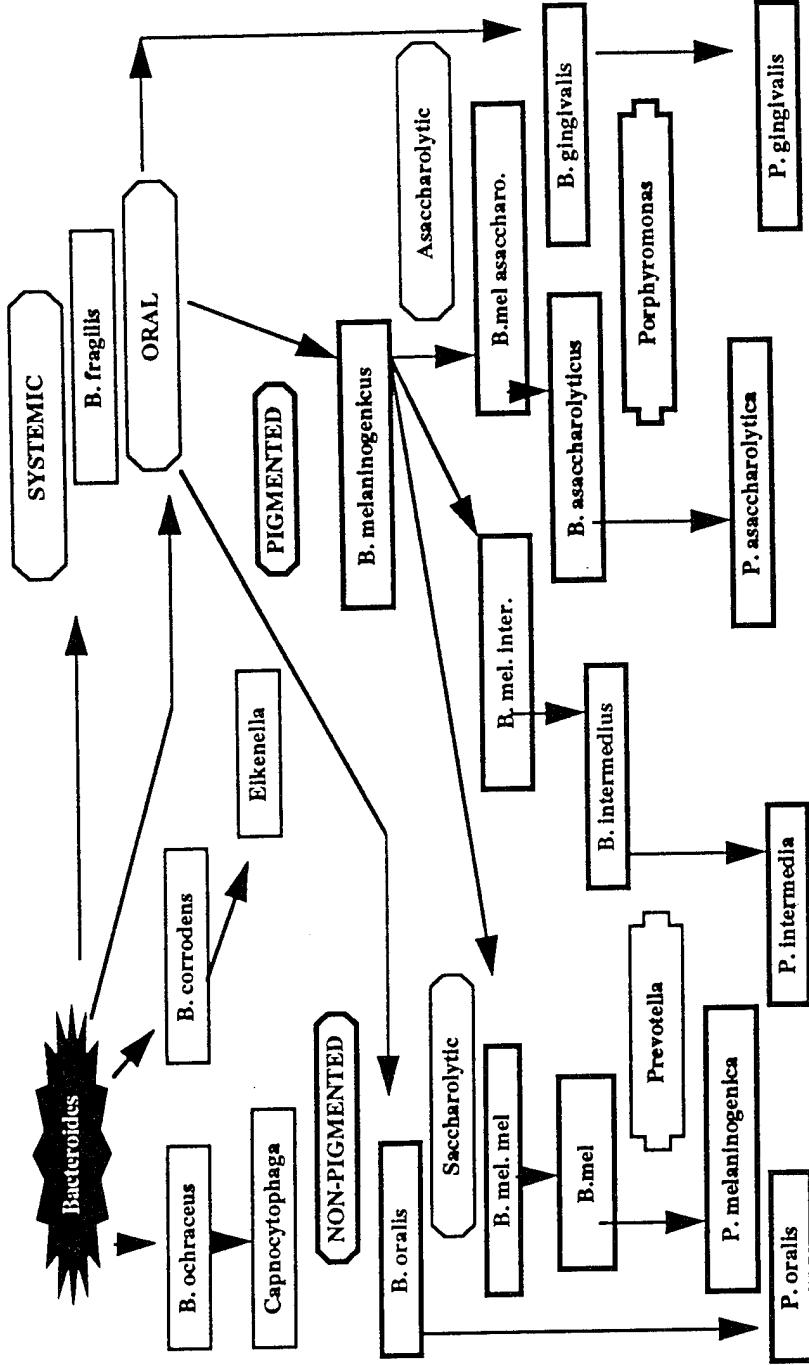
B. Facultative Gram Negative Rods: There are a large number of these in the oral cavity; many are capnophilic and until recently they were overlooked. Some examples include:

1. **Haemophili:** regularly present in dental plaque, saliva and on soft tissue surfaces. The species most commonly isolated from the mouth are *Haemophilus parainfluenzae*, *H.paraphrophilus*, and *H.segnis*. The cells are pleomorphic, nonmotile, and noncapsulated, forming mainly irregular filaments with a few bacillary forms.

2. **Eikenella corrodens:** small coccobacilli found in active periodontal pockets. This species is a facultatively anaerobic organism.

3. **Actinobacilli:** *A.actionmycetemcomitans* has been associated with Juvenile Periodontitis

Recent Name Changes For Bacteroides



4. Capnocytophagae: a group of fastidious, CO₂-requiring, gram negative, fusiform rods. They are characteristically described as gliding or surface translocating bacteria. These organisms are associated with juvenile periodontitis and possibly other forms of destructive periodontal disease.

C. Curved Gram Negative Rods

1. Wolinellae: genus recently proposed to accommodate organisms previously called *Vibrio succinogenes* and a new group of similar bacteria isolated from periodontal pockets, *Wolinella recta*. *Wolinella recta* has recently been switched to *Campylobacter rectus*

D. Gram Negative Cocci

1. Veillonellae: These are non-spore forming cocci that are among the most numerous oral bacteria. They comprise 5 to 10% of the cultivable flora of saliva and on tongue surfaces and tend to be parasitic in the mouth. *V.parvula* and *V.alacalescens* are common. They lack glucokinase and fructokinase and therefore cannot ferment sugars.

2. Neisseriae: Common species include *N.sicca* and *N.catarrhalis*. They are found on lips, tongue, cheek, plaque, and saliva and make up 1-2% of organisms. In contrast to some other organisms they do not seem to have any particular affinity for tissues and structures.

E. Gram-variable Filamentous Bacteria

1. Eubacterium: this genus contains a variety of anaerobic filamentous bacteria that are gram-variable. They have been shown to be important in pulpal and periapical infections and perhaps periodontal disease.

F. Spirochetes: common inhabitants. As gingival recession or pocket formation increases so do the number of spirochetes. Their number increases together with fusiforms.

III. Mycoplasma: regularly seen-generally in gingival area but also found at most other sites. Not well studied.

IV. Yeasts: *Candida albicans* is found in 10 to 80% of individuals.

V. Protozoa: protozoa are not uncommon. In particular *Entamoeba gingivalis* and *Trichomonas tenax* are found.

VI. Viruses: Except for *Herpes simplex* viruses seem to be transient.

VII. Prions

Neurodegenerative diseases of animals and humans including scrapie, bovine spongiform encephalopathy, and Creutzfeldt-Jakob disease are caused by unusual infectious pathogens called prions. Prions differ from bacteria, viruses, and viroids by their unprecedented structure and properties. There is no evidence for a nucleic acid in the prion, but diverse experimental results indicate that a host-derived protein called PrP^{Sc} is a component of the infectious particle.

SUMMARY OF ORAL MICROFLORA

Morphology	Gram Rx	Generic Name	Oral Site
Cocci	Positive	<i>Streptococcus</i> <i>S. faecalis</i> <i>S. mitis (mitior)</i> <i>S. mutans</i> <i>S. salivarius</i> <i>S. sanguis</i> <i>S. milleri</i> <i>Staphylococcus</i> <i>S. epidermidis</i> <i>S. aureus</i> <i>Peptostreptococcus</i> <i>Neisseria</i>	G M, S, D, T, G D T=S, M D, M, S, T, G G, D, T T, G S (FEW)
	Negative	<i>N. flavescens</i> <i>N. mucosa</i> <i>N. sicca (pharyngis)</i> <i>N. subflava</i>	
Anaerobic cocci	Negative	<i>Veillonella</i> <i>V. alcalescens</i> <i>V. parvula</i>	T=G=S
Anaerobic bacilli	Negative	<i>Bacteroides</i> <i>B. melaninogenicus</i> G <i>B. saccharolyticus</i> <i>B. oralis</i> G, D, T <i>Porphyromonas</i> G <i>Porphyromonas gingivalis</i> <i>Prevotella</i> G <i>Prevotella intermedia</i> <i>Eikenella</i> <i>Eikenella corrodens</i> <i>Capnocytophaga</i> <i>Capnocytophaga ochraceus</i> <i>Fusobacterium</i> G, D, T <i>F. nucleatum</i> <i>F. plauti</i> <i>Leptotrichia</i> <i>L. buccalis</i> <i>Selenomonas</i> <i>S. sputigena (Sprillum sputigina)</i>	
Non-spore forming rods	Positive	<i>Lactobacillus</i> species	G=S
Facultative anaerobic bacilli	Negative	<i>Haemophilus</i> <i>H. influenza</i> <i>H. parainfluenza</i> <i>H. (Actinobacillus)</i> <i>actinomycetemcomitans</i>	

Morphology	Gram Rx	Generic Name	Oral Site
Endospore-forming bacteria	Positive	Clostridium species	G
Actinomycetes and related organisms	Positive	<i>Corynebacterium</i> species Propionibacteria <i>P. acnes</i> <i>Actinomyces</i> <i>A. israelii</i> <i>A. naeslundii</i> <i>A. odontolyticus</i> <i>A. viscosus</i> <i>Arachnia propionica</i> <i>Bacteronema</i> <i>B. matruchotii</i> <i>Rothia</i> (<i>Nocardia</i>) <i>R. dentocariosa</i>	
Spiral and curved bacteria	Negative	<i>Campylobacter</i> <i>C. sputorum</i>	G
The spirochetes		<i>Treponema</i> <i>T. vincentii</i> <i>T. buccale</i> <i>T. denticola</i> <i>T. macrodentium</i> <i>T. mucosum</i> <i>T. orale</i> <i>T. scoliodontum</i>	G
Mycoplasma		<i>Mycoplasma</i> <i>T. salivarium</i> <i>T. orale</i>	G
Yeasts		<i>Candida</i> <i>C. albicans</i>	
Protozoa		<i>Entamoeba</i> <i>E. gingivalis</i> <i>Trichomonas</i> <i>T. tenax</i>	
Viruses		Herpes <i>Herpesvirus hominis</i>	

The letters designate the site(s) where the organism is usually found. If more than one site, they are given in decreasing order of preference unless present in approximately equal amounts. G, gingiva; D, dental plaque; T, tongue; M, mucosa; S, saliva

LECTURE 7: HOST-PARASITE INTERACTIONS (Microbial Pathogenic Mechanisms)

I. Ecology Of Infectious Disease

A. Bacterial Parasitism

The details of the multifactorial relationships between host and parasite are just beginning to be understood fully. The implications of this new knowledge will force health professions to reassess the definitions of a number of terms and concepts, such as disease, infection, pathogen, and virulence. This is underscored by the recent developments of new agents being identified as pathogens, agents previously considered benign becoming important sources of infections, and the emergence of entirely new diseases, for example Legionnaire's disease, Lyme disease, and acquired immunodeficiency syndrome (AIDS).

Parasite: An organism in or on another organism at whose expense it is maintained by obtaining food, shelter, or other advantages but which it does not usually destroy.

B. Microbial Relationships

The association between different microorganisms may be beneficial to both, may be beneficial to only one microorganism, or may be detrimental to one or more members of the community.

1. **Mutualism:** Achievement of a state of stability in the equilibrium between host and parasite will lead to permanent colonization of the organisms. Extremely complex relationships developed to keep non-resident flora,s out. The normal flora, initially derived by contamination from the moment of birth, may be defined as those organisms that are permanently established on and in mucous membranes and other superficial tissues. they participate in many host beneficial functions but may assume the role of a pathogen if entrance is gained to a normally sterile area of the body or a site where the organisms is not part of the established flora. This may occur as a result of a decrease in host resistance or invasive medical or dental procedures.

a. **Symbiosis:** beneficial relationships to one or more organisms. Examples would include the simultaneous growth of *Lactobacillus arabinosus* and *S.faecalis*. *L.arabinosus* produces folic acid and *S.faecalis* produces phenylalanine and therefore contributes to overall nutrition. In-turn, the host furnishes nutrients and a warm place to grow.

b. **Commensalism:** another example of stability between host and parasite or between one organism and another. Generally beneficial to only one microorganism. An example would be *B.melaninogenicus* when growing with diphtheroid organisms. *B.melaninogenicus* require vitamin K which is produced by the diphtheroids.

2. **Antibiosis:** relationships that may be detrimental to one or more members of the community. Examples would be the effects

of acids, formed by lactobacillus fermentation of carbohydrates or the production of antibiotics and bacteriocins.

3. **Synergism:** the result of several organisms producing a result that cannot be produced by individual growth.

Comment: Relationships are not permanent. Environmental factors and other factors will change it. Infectious disease is the result of an unsuccessful relationship between parasite and disease. The disease producing bacteria comprise a very small population, less than 200 species. The interaction can be described as colonization or infection. Infection and disease are not synonymous.

B. **The Life Cycle Of Parasites:** occurs in three stages

1. **Exposure:** usually occurs at mucous membranes in the oral cavity. Since the mucous membranes are continually exposed to parasitic bacteria, it is no coincidence that this tissue is bathed in SIGA which probably influences the rate of colonization. In addition, (1) organisms may be introduced into the body by a mechanical break or mediated through the bite of an infected insect of animal or minute fingernail and cuticle exposures in dentists. (2) A number of respiratory pathogens enter by inhalation. The dentist must be aware of the generation of dental aerosols by the high-speed drill. (3) The organism may be ingested directly with food or water and exert its virulence either through proliferation in the intestinal tract or by the production of toxic substances that may act on intestinal epithelium or be absorbed into the systemic circulation. (4) Phagocytes may carry obligate intracellular parasites through mucosa. and (5) Entry can occur by direct passage through epithelial cells.

2. **Colonization:** if the organism can withstand the initial host defenses it may colonize. Some organisms have specific binding mechanisms. *S.mutans* can bind to the tooth. The structures responsible for these activities are collectively called adhesins. These include fimbriae and pili.

3. **Exit:** to be successful a parasite must be able to exit. Disease-producing bacteria have not yet attained this state of parasitism.

II. **Etiology Of Infectious Disease**

Koch's Postulates:

1. The suspected organism should occur in association with a particular disease, however, it should be absent from a healthy host.
2. The organism must be isolated and grown in pure culture.
3. When this pure culture is introduced in a suitable laboratory animal, disease should occur.
4. The organism must be re-isolated from the diseased animal and the isolate shown to be identical with the isolate in step 2.

Molecular Koch's Postulates:

1. The phenotype, or property, under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species.
2. Specific inactivation of the gene(s) associated with the suspected virulence trait should lead to a measurable loss in pathogenicity or virulence.
3. Reversion or allelic replacement of the mutated gene should lead to restoration of pathogenicity.

A. Dental Caries: clearly an infectious disease of bacterial etiology but specific proof is slow. Koch's third postulate is difficult to prove.

B. Periodontal Disease: Evidence indicates that periodontal disease has a microbial etiology.

C. Diseases Of Multiple Etiology: although *S.mutans* is considered the prime cariogen in man, other oral bacteria can give rise to carious lesions in experimental animals. In periodontitis it is almost certain that several plaque bacteria contribute to the inflammatory process.

III. Determinants Of Bacterial Virulence: the pathogenic potential of bacteria is one of their most important aspects and one of the reasons bacteria have received so much study. A number of specific **virulence factors** are associated with the ability of certain bacteria to produce disease. Not all pathogenic bacteria have each of these factors or even a majority of them. Similarly, the expression of even one factor is subject to a series of genetic and environment control mechanisms.

-simply stated bacteria cause disease in two main ways: 1) by invasion of virgin tissue, and 2) by elaboration of toxins.

A. Invasiveness: Bacteria that are virulent due to invasive properties such as production of M protein, capsules, pili or ones that are taken up by phagocytes and survive

1. Extracellular Parasites:

- a. **Pili:** may prevent phagocytosis
- b. **Capsule:** not all are carbohydrates

2. Intracellular Parasites: examples are tubercle bacilli, brucella and listeria

3. Antiphagocytic factors: capsule, M-protein

4. Adherence: a fundamental prerequisite for bacterial pathogenesis is the ability of a bacterial cell to adhere specifically to surfaces of the host prior to actual tissue damage

B. Toxigenicity: generally disease is the result of the parasites ability to produce powerful pharmacologically active, diffusible proteins

1. Exotoxins: examples include diphtheria toxin, streptolysin O from the streptococci, leukocidin from staphylococci, and leukotoxin from *A.actinomycetemcomitans*.

2. Endotoxins: integral components from the outer membranes of gram negative cell walls. Lipopolysaccharides.

3. Invasins (Bacterial Enzymes)

a. **Hyaluronidase** -breaks down hyaluronic acid and facilitates spread of infection. Formed by oral streptococci. Is antigenic and immunologically specific for streptococci.

b. **Kinase**-causes the breakdown of blood clots and may aid the organism in the spread of infection

c. **Lecithinase**-combines with calcium, attacks phospholipids, and causes lysis of tissue cells, red blood cells, and leukocytes; also can inactivate enzymes dependent on lecithin

d. **Coagulase**-causes clotting, resulting in a fibrous covering about the microorganism or the lesion

e. **Collagenase**-destroys collagen fibers

f. **Chondrosulfatase**-hydrolyses chondroitin sulfate, a tissue-cementing polysaccharide

g. **Neuraminidase**-a tissue-degrading enzyme

h. **Hemolysin**-causes lysis of red cells

i. **Leukocidin**-destroys PMN's

j. **Diphosphopyridine nucleotidase**-associated with toxicity for leukocytes

k. **Necrotoxin**-kills tissue cells

l. **Streptodornase**-liquefies purulent exudates by dissolving DNA and RNA

INVASINS

Hyaluronidase	breaks down the intercellular substance, hyaluronic acid, and facilitates spread of infection through tissues
Kinase	causes the breakdown of blood clots and may aid the organism in the spread of infection
Lecithinase	combines with calcium, attacks phospholipids, and causes lysis of tissue cells, red blood cells, and leukocytes
Coagulase	causes clotting, resulting in a fibrous covering about the microbe or the lesion; protect microbe from phagocytosis
Collagenase	destroys collagen fibers
Chondrosulfatase	hydrolyses chondroitin sulfate, a tissue-cementing polysaccharide. Formed by several oral microbes
Neuraminidase	a tissue-degrading enzyme
Hemolysin	causes lysis of blood cells
Leukocidin	destroys PMN's. Formed by many streptococci
DPP nucleotidase	associated with toxicity for leukocytes
Necrotoxin	kills tissue cells
Streptodornase	liquifies purulent exudates

C. Role of Hypersensitivity in Virulence: subsequent introduction of the antigen results in an increased hypersensitivity reaction which can result in tissue damage.

D. Acquired Virulence Attributes Associated With Plasmids

1. the acquisition of pathogenic properties, such as toxin and new enzyme production
2. acquired ability to adhere to and colonize specific tissue surfaces
3. acquisition of multidrug-resistant trait
4. acquisition of the ability to form bacteriocins that may facilitate competitive colonization in infection
5. acquisition of new biochemical properties that may lead to incorrect identification of clinical isolates

LECTURE 8: MECHANISMS OF ORAL COLONIZATION AND THE MICROBIOLOGY OF DENTAL PLAQUE

Historically many terms have been used to describe the organic material which accumulates on tooth surfaces. Since caries and periodontal diseases usually develop in areas heavy of heavy plaque accumulations, documentation of microbial composition is critical to the understanding, treatment and prevention of these diseases.

Plaque: The soft, nonmineralized bacterial deposit which forms on teeth and dental prostheses that are not adequately cleansed. Microscopically plaque is simply confluent colonies of microorganisms connected by a matrix consisting of bacterial and salivary polymers. Dental plaque may also be defined in terms of its location (**supragingival** or **subgingival**), its properties (**adherent** or **nonadherent**), or its pathogenic potential (**cariogenic** or **periodontopathic**). These classifications are not mutually exclusive. In general, supragingival plaque is adherent and contains a predominantly gram-positive flora, which often contains cariogenic organisms. In contrast, subgingival plaque is composed predominantly of gram-negative organisms, is less adherent than supragingival plaque, and contains periodontopathic organisms.

I. Ultrastructure and Gross Morphology: The tooth's surface is covered by several layers of organic material. The innermost layer is the acquired enamel pellicle, an adherent organic coating of the tooth derived from saliva. The next layer is dental plaque and the outermost layer is called **material alba** which is a covering of loosely adherent material consisting largely of ingested food. **Calculus** may be formed within dental plaque and is the hard mineral substance strongly adherent to tooth structure due to dental plaque that has become calcified.

A. Retentive and Nonretentive sites: areas subject to the normal oral cleansing activities of saliva are nonretentive and also include smooth surfaces. Retentive sites are those protected from cleansing and include pits and fissures.

B. Morphology: Plaque morphology varies relative to individual, location, and age. With smooth surface plaque (nonretentive), the bacterial succession results in a layered structure. After pellicle formation colonies can be found in the gingival margin and these eventually merge to form a condensed bacterial layer. Although cocci or coccoid microorganisms predominate (*Neisseria* and *streptococci*, with *streptococci* comprising up to 95% of the total cultivable flora after 24 hours accumulation) at this stage, filamentous cells may also colonize the pellicle in bundles often oriented perpendicular to the enamel (**test-tube brush forms**). Few gram-positive rods are found in the early stages of plaque development, although strains of *lactobacilli*, *actinomycetes*, and *Rothia dentocariosa* have been isolated. Other than *Veillonella*, anaerobic species do not appear until the later stages of plaque development. Interspersed between the microcolonies is a **fibrillar matrix**. There is generally an accumulation of morphologically similar bacteria in colonylike aggregates. Typical of this is the palisadelike structure formed by the filamentous cells. Mixed bacterial aggregates with characteristically **corn cob** morphology can also be identified. With time, the pellicle is degraded and bacteria can have direct contact with the crystalline enamel.

surface.

Plaque does not appear to be a mass of passively acquired material although fissure plaque can be. Plaque at the fissure apex is similar to smooth surface plaque with merging into a condensed layer. The palisadeing microbial aggregates and branching filaments are absent. Also deep within the fissure there are more dead cells and ghost like cells wall structures.

Plaque below the gingival margin is associated with the accumulation of supragingival plaque. As certain microflora at the gingival margin produce inflammatory changes in the gingiva, the susceptibility of subgingival space for bacterial accumulation increases. Once bacteria have gained access to this space. They are protected from the usual cleansing mechanisms of brushing and flossing. Bacteria that gain access to this space tend to be fastidious anaerobes.

C. Plaque Initiation:

1. Early colonization: *S. sanguis*

2. Role of saliva: chemical analysis of the pellicle shows a composition similar to that of saliva. Salivary agglutinins are also present.

3. Specificity:

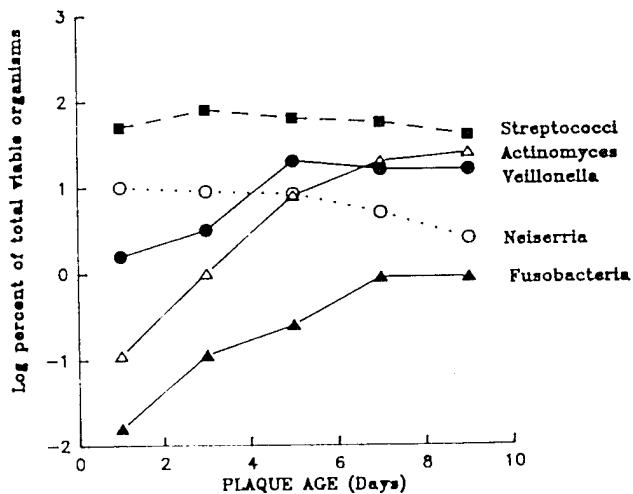
a. initial colonization

b. salivary receptors

c. adhensins

d. functional domains of both the adhesins and receptors that interact.

D. Plaque Maturation: a number of investigators have documented the changes that occur as plaque ages. Bacterial changes are shown in the following figure.



E. Composition:

Chemical Composition Of Dental Plaque

Component	Wet Weight (%)	Dry Weight (%)
Water	80	
Solids	20	
Protein		40-50
Carbohydrate		13-18
Lipid		10-14
Inorganic		5-10

1. Protein content: proteins are a major component but proteins of the acellular fraction is low. These proteins are probably host related and come from saliva and serum. Salivary proteins such as amylase, lysozyme, lactoferrin, lactoperoxidase, SIGA, and proteases are found. Bacterial derived hyaluronidase, collagenase, glucosyltransferase have also been demonstrated.

2. Carbohydrate content: carbohydrates as poly and oligosaccharides are very heterogeneous. Homopolysaccharides are synthesized by plaque bacteria.

3. Lipid content: not much is known in this area. Phospholipids are made derived from host or bacteria. Lipids may participate in tissue mineralization due to affinity to bind ions such as calcium or phosphate to generate calculus.

4. Inorganic content: phosphate, calcium, and fluoride is found in higher concentration than in saliva.

5. Bacterial composition: approximately 200 to 300 species of bacteria reside as part of the dental plaque community. For each milligram of plaque there are about 10^8 bacteria. Because an average individual's mouth contains from 20 to 100 mg of plaque, there can be as many as 10^{10} bacteria in the plaque of a single individual.

a. **Bacterial succession:** generally a switch from gram positive to gram negative organisms and from aerobes to anaerobes.

b. **Specificity of bacterial attachment:** bacteria must attach or they will be washed away by normal salivary flow. When all the sites on the tooth are filled, the incoming bacteria must recognize and adhere to already attached bacteria.

COAGGREGATION BETWEEN ORAL BACTERIA

Gram-positive	Gram-positive
<i>Streptococcus sanguis</i> and/or	<i>Actinomyces viscosus</i> <i>Bacterionema matruchotii</i> <i>Rothia dentocariosus</i> <i>Actinomyces naeslundii</i>
<i>S. mitis</i> , <i>S. gordonii</i>	<i>A. naeslundii</i> <i>A. odontolyticus</i> <i>Bacterionema matruchotii</i> <i>Propionibacterium acnes</i>
Gram-positive	Gram-negative
<i>Streptococcus sanguis</i> (<i>S. anginosus</i>)	<i>Fusobacterium nucleatum</i> <i>Bacteroides loescheii</i>
<i>Streptococcus</i> sp. or	<i>Porphyromonas</i> and <i>Prevotella</i> sp. <i>Capnocytophaga</i> sp.
<i>Actinomyces</i> sp. <i>A. israelii</i>	<i>Fusobacterium nucleatum</i> <i>Eikenella corrodens</i> <i>Veillonella</i> sp. <i>Bacteroides loescheii</i>
Gram-negative	Gram-negative
<i>Prevotella melaninogenica</i>	<i>F. nucleatum</i>

II. Factors Influencing The Composition Of Dental Plaque: a variety of factors causes plaque to be a complicated ecosystem.

A. Host Factors: *B.melaninogenicus* and spirochetes are generally absent from young individuals. *A.naesilundii* predominates. *A.viscosus* is seen in teens and adults. Physiologic changes occurring during aging may affect receptor sites of metabolic factors required for colonization. Changes in sex hormones. Disease states resulting in inflammation can also be involved. Dietary factors are also important. Food is eliminated quickly from the mouth but in some places it persists. Diets supplemented with sucrose can cause increases in some bacteria.

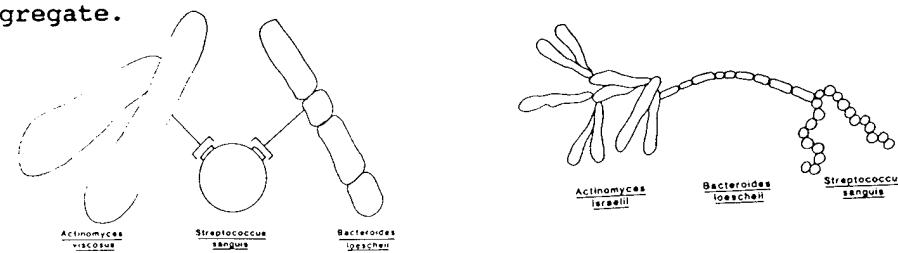
III. Interactions Among Plaque Bacteria

A. Mutacins: a kind of bacteriocin that inhibits gram positive bacteria. It may be ecologically advantageous in a microenvironment.

B. Coaggregation: the recognition between surface molecules on two different bacterial cell types so that a mixed-cell aggregate is formed. It is likely that most if not all human oral bacteria participate in coaggregation, and that these interactions have a major role in bacterial adherence and colonization on host surfaces. Co aggregation appears to be mediated by specific recognition between complementary lectin-carbohydrate molecules on the participating partners.

Principles Of Coaggregation:

1. Coaggregation is a result of recognition between accessible surface components on bacterial partners. Cell walls and isolated adhesin or receptor molecules mimic whole cells.
2. Adherence between partners is tenacious.
3. Coaggregation occurs between specific partners. Cell viability is not required.
4. Coaggregation specificity is a stable property. Older stock cultures maintained in various laboratories exhibit coaggregation properties identical to those of freshly isolated strains of the same species.
5. Different kinds of surface components mediate coaggregations. Carbohydrates, fimbriae, and outer-membrane proteins have been identified.
6. Simple sugars such as lactose often inhibit coaggregations.
7. A partner that is recognized by two or more noncoaggregating cell types can be involved in (a) competition when the cell types interact with the same component on the common partner, or (b) bridging when the cell types interact with different components on the common partner.
8. Multigeneric aggregates (bridging) are composed of a network of coaggregating pairs acting independently. No unexpected surface interaction is detected when a coaggregating pair becomes part of a multigeneric aggregate.



PLAQUE FORMATION AS A SPECIFIC EXAMPLE OF ECOLOGICAL SUCCESSION

Characteristics	Early supragingival Plaque	Mature supragingival Plaque	Mature subgingival Plaque
Gram reaction	+	+/-	Dominated by -
Morphotypes	Cocci, branching rods	Cocci, branching rods, filaments, spirochetes	Dominated by rods and spirochetes
specific organisms	<i>S.sanguis</i> , <i>A.viscosus</i>	<i>Actinomyces</i> , <i>Veillonella</i> , <i>Nocardia</i> , <i>Bacterionema</i>	<i>Bacteroides</i> , <i>Prevotella</i> , <i>Porphyromonas</i> , <i>Fusobacterium</i> , <i>Treponema</i>
Energy metabolism	Facultative	Facultative with some anaerobes	Dominated by anaerobes
Energy sources	Carbohydrate fermentation	Carbohydrate fermentation	Proteolysis
Motility	No, firmly attached	No, firmly adherent to plaque matrix	Adherence less pronounced with many motile forms
Toleration by host	Generally well	Can cause caries and gingivitis	Can cause gingivitis and periodontitis

LECTURE 9 : MICROBIOLOGY OF DENTAL CARIES

Caries, derived from the Latin word *cariosus*, meaning decay or rotten, is the localized destruction of smooth, pit and fissure enamel, as well as dentin and cementum of the teeth. Plaque formation on teeth is a prerequisite for development of dental caries. In the development of plaque on the tooth surface, certain oral streptococcal species appear to be of central importance. Of the principal oral streptococci, which include *S. mutans*, *S. sanguis*, *S. mitior*, *S. milleri*, and *S. salivarius*, the first four species comprise a significant percentage of dental plaque and contribute to its development.

In the context of oral microbiology and infectious disease dental caries is an **endogenous bacterial disease**. Caries involves bacteria that have latent pathogenic potential and which are indigenous to and ubiquitously found in the human oral cavity. The disease results from the development of conditions where the resistance of the host is lowered and specific bacterial strains can proliferate to elevated levels and express detrimental traits. The challenge to oral microbiologists and other dental scientists is to understand how and why this transition from a friendly symbiotic state to a harmful relationship takes place at specific times and at unique locations.

I. The Carious Process

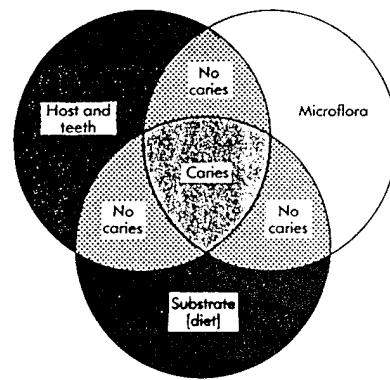
A. Caries Mechanisms: chemical analyses have demonstrated that the main process occurring in caries development is one of demineralization followed by replacement of the dissolved mineral with loosely bound water.

1. Acidity: the paramount chemical fact about the carious lesion is that its prevailing acidity is sufficient (Ph 5.2 or lower) to account for demineralization. However, the entire sequence of events seen cannot be reproduced by the action of acids alone. Changes in the organic phases (collagenous phase of dentin) must be attributed to bacterial invasion.

2. Proteolysis: although the weight of evidence favors acceptance of the chemoparasitic theory, others have been proposed. The proteolytic theory states that the protein fraction of enamel is initially attacked by bacteria and the destruction of the mineral fraction of teeth follows. The collagenous matrix is resistant to the degree of acidity that prevails in dental caries. The organic phase of dentin must be demineralized to be susceptible to degradative reactions. No one has degraded tooth protein without demineralizing it.

II. Etiology Of Caries

A. A Multifactorial Infectious Disease: although the cause of dental caries is complex, it has been shown that there is an interrelationship among host, diet, bacteria, and time.



1. Host Susceptibility: variation in host susceptibility may be due to genetic factors, age of the tooth, tooth composition, salivary flow and composition, and immune status.

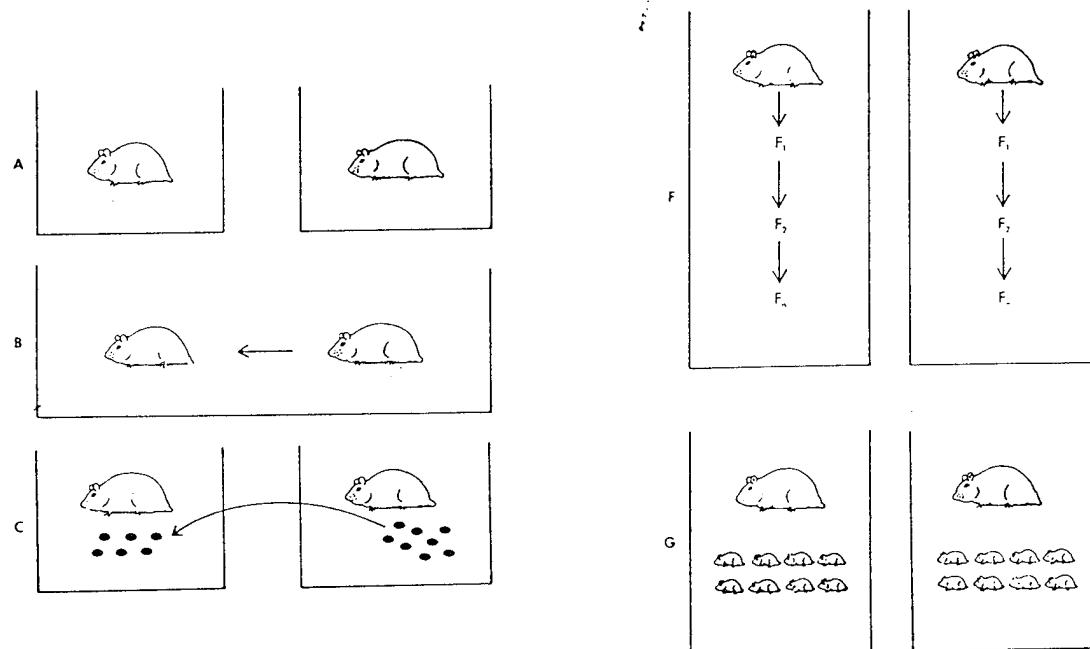
2. Dietary Factors: dietary factors that contribute to the development of dental caries are centered primarily about various sugars, especially sucrose. Most studies support the statement that sugar is the most important dietary factor leading to the development of caries. Sucrose offers acidogenic bacteria in plaque an opportunity to form organic acids.

3. Bacterial Etiology: it now is very clear that bacteria are involved in caries development and the specific organisms involved will be dealt with below:

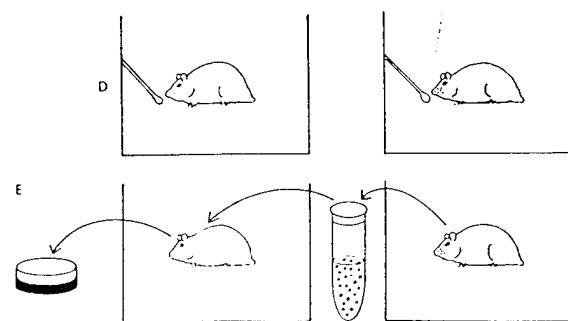
4. Time: the classic triad of host, diet and microbial factors needs to be joined by a time factor since this parameter emphasizes the need for a simultaneous interaction of the various contributions.

B. The Infectious Nature of Dental Caries: a long-standing tenet has held that human genetic factors, expressed as "susceptibility" or "resistance" to caries (often clinically stated as possession of either "soft" or "hard" teeth) was central to caries determination. No evidence, however, indicates differences in hardness between noncarious teeth of individuals without caries and those of individuals with caries. Nonetheless, some human families have a very high experience of caries, whereas others have a relatively low experience, and the experience of caries of one family member may be at variance with that of others in the family. It is well known, however, that family patterns of other diseases may be attributable to either genetic or environmental factors, especially infection and dietary status.

1. Genetic or Infectious: the cause of dental caries was greatly clarified by two kinds of observations made in the 1950s and 1960s: (1) that germ-free rats develop no carious lesions, regardless of their diet, unless they are inoculated by certain microorganisms; and (2) that caries is transmissible in hamsters, rats, gerbils, and a variety of other animal models of human caries.



A, Normally albino hamsters develop no carious lesions when consuming sucrose whereas golden hamsters develop severe lesions a few weeks after sucrose is provided to weanlings. **B,** Caging of albinos together with goldens results in development of caries in the albinos as well as in the goldens. Penicillin blocks disease. **C,** Transfer of feces from goldens to cages or mouths of albinos transmits the disease and penicillin blocks disease. **D,** Transfer of plaque swabbings from goldens to mouths of albinos transmits the disease; penicillin blocks disease. **E,** Inoculation of pure culture isolates of streptococci found in both the feces and the plaque of goldens to the mouths of albinos, which were previously free of these bacteria, transmits the disease. These streptococci can now be recovered from the feces and plaque of the disease-affected albinos; penicillin protection of the albinos blocks transmission. **F,** Progeny of caries-affected goldens or of caries-unaffected albinos are like their parents. Penicillin administration to goldens blocks intergenerational transmission of disease. **G,** Caesarean delivery with cross-suckling results in the development of caries in the albino pups but not in the golden pups.



2. Specific Versus Nonspecific Hypothesis: An area of importance in caries etiology, which is still controversial, is whether the microbial etiologic component is specific or nonspecific. Specific etiology means that a single microorganism causes dental caries. Nonspecific etiology means that several microorganisms cause dental caries. Cross-sectional and longitudinal studies in humans have shown that *Streptococcus mutans* is associated with dental caries, but in a smaller but significant number of cases, this organism was not detected when caries was present. Conversely, *S. mutans* was found on tooth surfaces when caries did not occur. Proponents of the nonspecific hypothesis contend that any acidogenic microorganism in dental plaque contributes to the causation of dental caries.

3. *Streptococcus mutans* and Dental Caries: The clear association of *S. mutans* with human dental caries, and the demonstration that these isolates are fully virulent in animal models, suggest, but do not prove, that *S. mutans* is of major etiologic significance in human dental caries.

The facts that indicate that streptococci, especially *S. mutans*, are etiologic agents are as follows:

- a. discovery of mutans streptococci in human carious lesions (Clark, 1924)
- b. rediscovery of mutans streptococci in man and association with carious lesions (1960s); certain streptococci, especially *S. mutans*, have been isolated from human carious lesions and from plaques of individuals with high caries activity. In people with low caries activity, *S. mutans* is found less frequently and in lower numbers.
- c. human isolates are cariogenic in various animal models initially free of mutans streptococci (Koch's postulates)
- d. when preventative measures were used to reduce *S. mutans* in mothers of infants, there was a decrease of *S. mutans* in both mothers and infants when compared to controls
- e. *S. mutans* causes caries in experimental animals
- f. Studies of an experimental caries vaccine in animals have shown that a vaccine derived from *S. mutans* reduces caries
- g. Temporal correlation between colonization of tooth sites and of pooled plaque (and saliva)
- h. "Great Lakes" study: further destruction of genetic resistance hypothesis

Microorganisms most strongly implicated in human dental caries induction and progression

Microorganism	Induction (sites)	Progression
Mutans streptococci <i>S. mutans</i> <i>S. sobrinus</i> <i>S. rattus</i> <i>S. cricetus</i>	4+ (ss, pf, and rs)	Possible
<i>S. sanguis</i>	Weak (pf)	Doubtful
<i>S. mitis/S. mitior</i>	Weak (pf)	Doubtful
<i>S. anginosus</i> (<i>S. milleri</i>)	Weak (pf)	Doubtful
Enterococci	Weak (pf)	Doubtful
<i>A. viscosus</i>	Possibly strong (rs only)	Possible
<i>A. naeslundii</i>	Nil	Possible
Lactobacilli	Weak (ss, pf) Possibly strong (rs only)	Probable
Unident facultative and anaerobic cells	Nil	Probable

- i. Colonization of nonshedding oral surfaces, not mucosa ("Womb to tomb" study)
- j. Mother's infection level is predictor of her child's date of infection and caries experience, matrilineal transmission
- k. Bacteriocin pattern identity of mother's and child's mutans streptococci
- l. *S.mutans* produces insoluble extracellular polysaccharides from sucrose that allow it to stick to the teeth and form larger amounts of plaque
- m. pH levels of 5.0 or lower may persist in caries-active plaques for several hours. This indicates that the microbial acid production is maintained in the presence of plaque and salivary buffer systems. *S. mutans* produces acid at pH 5.0

Relationship Between *Streptococcus mutans* And Human Dental Caries

	Pit and Fissure Caries Studies		Pooled Plaque Studies	
	Active Caries	No Lesions	Rampant Decay	Caries-Free
Active Caries Studies	71% of lesions contained greater than 10% <i>S.mutans</i>	70% of these studies has no <i>S.mutans</i>	<i>S.mutans</i> comprised 10% of the flora in 65% of cases	In 40% of cases no <i>S.mutans</i> was detected
Pre-Caries Studies	Association of <i>S.mutans</i> with Caries-Prone Toothsites (195 teeth sampled)			
	Teeth Developing Lesions		Teeth Remaining Caries-Free	
	42		153	
	37/42 had high counts of <i>S.mutans</i> before and during attack		Subjects divided in high and low caries activity. <i>S.mutans</i> low or absent in LCA and high in HCA.	

Comment: *S.mutans* has nearly every feature you could desire in a microbe designed to cause caries-lives only on teeth, utilizes sucrose in clever ways to foster colonization while using other sugars to make acid, resists attempts at removal, is selective.

3. Other Cariogenic Bacteria and Problems With Identifying "THE" Etiologic Agent: Although most investigators believe that *S. mutans* is the primary etiologic microorganism for typical coronal caries, lactobacilli should not be ignored. They should be considered to have a secondary or opportunistic role. The implication of lactobacilli as etiologic agents is supported by both experimental evidence and clinical observation.

- a. lactobacilli causes rampant dental caries in germ-free rats.
- b. artificial caries in human teeth was induced by lactobacilli
- c. when carious areas were restored in humans or if there was a spontaneous regression of caries, lactobacilli were no longer observed.
- d. a longitudinal study in humans has shown that lactobacilli are present at a sound, susceptible site before the clinical observation of caries.

In addition, with deep dentinal lesions, actinomyces, arachnia, bacillus, bifidobacterium and eubacterium may also be involved. Actinomyces also seem to be involved in root surface lesions.

III. Characteristics of *Streptococcus mutans*

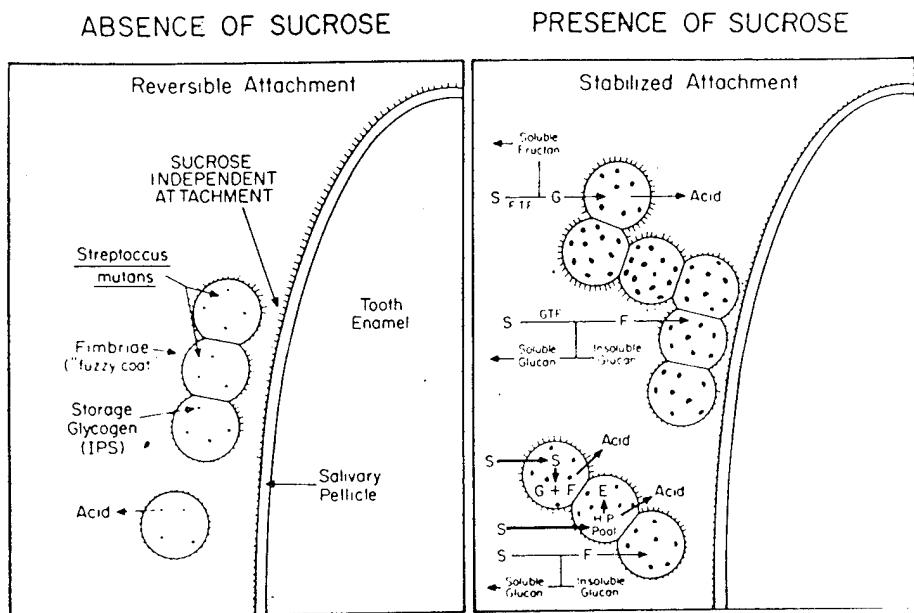
A. Classification: Gram-positive cocci occurring in chains, nonmotile, catalase negative, alpha or gamma hemolysis. Seven different serotypes (a-g) have been identified with Group c the most prevalent. Of these, 5 biotypes have been identified.

B. Cell Wall Macromolecules: main interest is on surface structures of importance in adherence.

1. **Cell wall carbohydrate:** the immunodominant carbohydrate for each serotype is known. They do not cross-react.
2. **Peptidoglycan:** responsible for shape and rigidity of the cell
3. **Lipoteichoic acid:** all have lipoteichoic acids known to exhibit immunologic effects including immunogenicity. It protrudes through the cell wall and probably also is involved in attachment.
4. **Surface proteins (fimbriae):** may also be involved in attachment.

C. Adherence and Plaque Formation: adherence to the tooth surface involves two stages: an initial reversible interaction between the cell and saliva-coated tooth surface and an irreversible stage mediated by water insoluble glucan.

1. Sucrose-dependent Virulence: One of the more unique features of *S. mutans* is the ability to utilize dietary sucrose to enhance its colonization of the oral cavity. The production of extracellular glucans is considered to be the critical reaction in the oral accumulation and cariogenicity of this bacterium.



2. GTF-glucan-induced Adherence: glucosyltransferase conversion of sucrose to polyglucans.

D. Acid Production: homofermentative producing lactic acid from pyruvate

E. Prevention of *Streptococcus mutans* Induced Dental Caries: needs to be carefully considered-we are just beginning to appreciate the development and immunological consequences of the several grams of oral bacteria which humans swallow each day. Some areas to consider include antimicrobial agents and antibiotics, immunization, modification of cariogenic bacteria, and diet modification.

LECTURE 10: MICROBIOLOGY OF PERIODONTAL DISEASE

There is substantial evidence that bacteria are the essential etiologic agents in gingivitis. By implication from animal studies and results of human clinical trials which control the bacterial population, there is presumptive evidence that bacteria are also etiological operative in periodontitis. A review of the microbiologic aspects of periodontal disease while not producing a total understanding of the role of microbes in the pathogenesis of disease will indicate certain organisms that do play a major role.

I. Microbiology Of Periodontal Disease: Evidence for a bacterial etiology of periodontal disease is as follows:

- a. gingivitis develops with increasing plaque accumulation
- b. the inflamed tissue in gingivitis is restored to its healthy state by plaque removal
- c. certain antibacterial agents prevent gingivitis and may aid in treatment of periodontitis
- d. laboratory animals infected experimentally with specific oral bacteria develop periodontal disease
- e. gnotobiotic animals do not develop periodontal disease

A. **Microbial Etiology:** although it is clear that inflammatory periodontal diseases are caused by microorganisms, it is also clear that these diseases differ from most other infectious diseases in that gingival and periodontal disease sites are associated with large numbers of different microorganisms. This has resulted in the development of two hypotheses related to the microbial etiology:

1. **Nonspecific Plaque Hypothesis:** suggests that periodontal disease is caused by a quantitative increase in the amount of plaque rather than by the presence of certain microorganisms in the plaque.

2. **Specific Plaque Hypothesis:** suggests that periodontal disease is caused by the presence or the concentration of a specific microorganism(s) in plaque.

B. **Microbial Flora Associated With Periodontal Health:** the healthy gingival sulcus harbors a scant microflora dominated by gram-positive organisms and facultative anaerobic species. Spirochetes and motile rods make up less than 5% of the healthy flora. Specific organisms include:

Streptococcus sanguis
Actinomyces viscosus
Actinomyces naeslundii
Veillonella parvula
and low levels of *Fusobacterium* and *Prevotella*

C. **Microbial Flora Associated With Gingivitis:** Sites of gingivitis contain 10 to 20 times more organisms than healthy sites. All of the species present in healthy sites seem to be present in gingivitis. *Actinomyces* and *streptococci* each constitute 25% of the flora. Gram negative anaerobic rods now comprise another 25% of the flora. The majority of these species include *Prevotella (Bacteroides) intermedia*,

Fusobacterium nucleatum and other *Bacteroides*. *Veillonella*, *Wolinella*, *haemophilus* and *spirochetes* are also found. *Pregnancy gingivitis* has been associated with "blooms" of *P.intermedia*.

D. Microbial Flora Associated With Periodontal Diseases:

Although periodontal disease is associated with a complex microflora, few species may be responsible for the transition of gingivitis lesions into periodontal lesions. There seems little doubt that gram negative anaerobic bacteria play a major role in the pathogenesis of human periodontal diseases. Organisms occupying prominent positions include:

Porphyromonas (Bacteroides) gingivalis

Prevotella (Bacteroides) intermedia

Actinobacillus actinomycetemcomitans

Eikenella corrodens

Campylobacter (Wolinella) recta

Fusobacterium nucleatum

Treponema denticola

Capnocytophaga species

1. Microbial Flora Associated With Categories Of Periodontal Disease

- a. Severe adult periodontitis

P. gingivalis

- b. Localized juvenile periodontitis

Actinobacillus actinomycetemcomitans

- c. Advanced periodontitis in juvenile diabetics

Capnocytophaga

- d. Pregnancy gingivitis

P. intermedia

- e. ANUG

P. intermedia and *spirochaetes*

II. Virulence Factors: As has been indicated before, microbial factors in the development of an infectious disease include colonization of the host, growth of the organism, penetration of tissues by the microorganism or products of the microorganism, and direct bacterial or immunologically induced damage of the tissue. Virulence of an organism is a multifactorial property influenced by the inherent pathogenic potential of the organism, the habitat of the organism, and a number of host determinants. Also, although a certain microbial property may be necessary for virulence, the mere possession of the property is not sufficient to make an organism virulent. In order to produce periodontitis, an organism must (1) establish close proximity to periodontal tissues, (2) avoid being swept away by saliva or GCF, (3) acquire essential nutrients for growth, (4)

resist bacterial antagonism and host defenses, and (5) be able to induce periodontal tissue destruction. Some of the microbial virulence factors of possible importance in the etiology and pathogenesis of destructive periodontal disease are indicated below:

Selected microbial virulence determinants in periodontal disease

Colonization	Evasion	Tissue Destruction
Fimbriae	Leukoaggressins:	Collagenase
Capsule	Chemotaxis inhibitors	Hyaluronidase
Lipopolysaccharide	Phagocytosis inhibitors	Sulfur compounds
Microbial antagonism	Killing inhibitors	Bone resorption
Microbial synergism	Proteases against:	Acid phosphatase
	Immunoglobulin	Epithelial cell toxin
	Complement	Fibroblast inhibitors
	Plasma proteinase cascade components	Endothelial cell toxins
	Fibrinolysin	
	Proteinase inhibitors	
	Siderophores	
	Cytotoxins	
	Surface Fc-receptor	
	T-cell suppressor cell stimulation	
	Antigenic shift	

A. Adherence Factors: *S.sanguis* and *A.viscosus* play important roles as very early colonizers. These as well as other attached cells, proliferate, and the resulting chemical and physical changes with the developing plaque influence attachment and proliferation of similar or additional types of bacteria. As the plaque bacteria proliferate, the microbial mass extends in all directions. Besides being responsible for the early plaque development, certain species of *Streptococcus* and *Actinomyces* can undergo coaggregation and bind gram-negative organisms. *Actinomyces* can bind *Fusobacterium nucleatum*, *Eikenella corrodens*, *Veillonella parvula*, *Capnocytophaga ochracea*, *Porphyromonas gingivalis*, *Bacteroides intermedius*, and *Bacteroides melaninogenicus*

B. Growth And Penetration Of Tissues: Bacterial penetration of the periodontal tissues has long been thought to occur only in acute necrotizing ulcerative gingivitis. There have been less convincing reports concerning other periodontal diseases. Within the last few years, several studies have demonstrated the presence of some bacteria within the tissues during adult periodontitis and juvenile periodontitis.

MICROBIAL SPECIES ASSOCIATED WITH PERIODONTAL DISEASES

Organism	GI	PG	AP	LJP	HIV	I	ANUG
<i>Actinobacillus actinomycetemcomitans</i>	-	-	++	+++	+	+	-
<i>Porphyromonas gingivalis</i>	-	-	+++	-	+	+	-
<i>Prevotella intermedia</i>	++	+++	++	+	++	++	+++
<i>Bacteroides forsythus</i>	-	-	++	-	?	?	?
<i>Fusobacterium sp.</i>	+++	++	++	-	+	+	++
<i>Peptostreptococcus micros</i>	-	-	+++	-	++	++	?
<i>Wolinella recta</i>	+	+	++	+	+++	+	+
<i>Treponema denticola</i>	+	+	++	-	++	+	+++
Enteric rods/pseudomonads	+	?	+	-	++	+	?
<i>Streptococcus species</i>	+++	++	-	-	-	-	+
<i>Actinomyces species</i>	+++	++	-	-	-	-	-
Spirochetes	+	+	++	-	++	+	+++

GI, gingivitis; PG, pregnancy gingivitis; AP, adult periodontitis; LJP, localized juvenile periodontitis; HIV, HIV-periodontitis; I, perimplants; ANUG, acute necrotizing ulcerative gingivitis

C. Periodontal Tissue Destruction: the development of periodontitis involves breakdown of the collagenous periodontal ligament and supportive alveolar bone. Periodontal tissues may be destroyed either by direct release of damaging bacterial products or by microbially induced destructive host reactions. Microbial products directly harmful to the host include enzymes, toxins, and metabolites.

1. **Toxins:** The tissue destruction is due not only to harmful extracellular products of plaque bacteria but also to potentially harmful antigens or endotoxins in the cell walls of the bacterial cells. The only exotoxin thus far thought to be related to periodontal disease is the leukotoxin produced by *Actinobacillus actinomycetemcomitans*. Endotoxin may also be related to posttreatment healing in periodontitis.

2. **Enzymes:** Enzymes such as collagenase, hyaluronidase, phospholipase A, lecithinase, phosphatase, proteases, and neuraminidase are found among the periodontitis associated bacteria.

3. **Bacterial Metabolites:** several suspected periodontal pathogens release volatile sulfides (methylmercaptan, hydrogen sulfide, dimethyl sulfide) that are inhibitory to collagen and noncollagenous protein synthesis.

D. Indirect Tissue Destruction: the host defense system is a two-edge sword. On one hand, it protects against microbial infections and tumor development; on the other hand, it is capable of causing severe tissue injury. LPS of *P.gingivalis* and other bacteria stimulates *in vitro* the release of prostaglandin E₂ and interleukin-1 β from macrophages, all of which demonstrate bone resorbing potential as well as various inflammation-inducing activities. Butyric and other short-chain fatty acids may also stimulate interleukin-1 β production, as well as suppress the production of T lymphocytes. A number of immunopathological mechanisms may play roles in tissue destruction and this will be discussed during the Immunology Course.

E. Bone-Resorbing Factors: Agents like lipopolysaccharides and teichoic acid are known to stimulate the production of osteoclast activating factor.

F. Other Factors: other materials that may act as virulent factors include antigens causing adverse immunologic reactions, bacterial substances that activate the complement system and immunosuppressive factors.

II. Characteristics and Pathogenic Potential Of Selected Periodontal Pathogens

A. *P. gingivalis*-anaerobic Gram-negative bacilli, black pigmented non-fermenter. *P.gingivalis* possesses some of the highest virulence potential of any oral organism tested thus far.

1. **Disease Potential:** oral, pulmonary, obstetric and gynecologic infections

2. Colonization:

- Attachment: pili, long fibers, capsule, vesicles, LPS
- Growth stimulatory: naphthoquinone, estradiol, progesterone succinate, hemin
- Growth-inhibitory: streptococci

3. Evidence:

- Inhibition of PMN: chemotaxis inhibitors, phagocytosis and intracellular killing
- C' mediated killing
- Endotoxicity: weak
- Immunoglobulin proteases: degrades IgA, IgG
- Fibrinolysin: strong activity and may invade the gingival connective tissue in part because no effective fibrin barrier is formed around the infecting organism
- Superoxide dismutase: may split PMN derived hydrogen peroxide and superoxide anions and enable the organism to resist oxygen dependent intra-leukocytic killing

4. Tissue Destruction

- Abscess-former: strong
- Enzymes: collagenase, trypsinase, gelatinase, aminopeptidase, phospholipase, alkaline phosphatase, acid phosphatase, DNAase, RNAase
- Toxic factors: epitheliotoxin, fibroblast growth inhibitors, endotoxins, LPS induced bone resorption, acids

B. *Prevotella intermedia*: very similar in characteristics to *P. gingivalis*

C. *Actinobacillus actinomycetemcomitans*: this is a small, non-motile nonencapsulated, Gram-negative, coccobacillus. It grows best on serum or blood agar in an atmosphere of 10% carbon dioxide

1. **Disease Potential:** most clinical isolates of A.a. have been from infected blood and bone. Some times mixed with *Actinomyces*. The apparent etiology of some cases is related to impaired host defenses in such diseases as malignant lymphoma and leukemias. A number of cases of subacute bacterial endocarditis have also been described.

2. Colonization:

- Attachment: pili, long fibers, capsule, vesicles, LPS
- Growth inhibitory factors: streptococci

3. Evidence: only oral bacterial species with leukotoxin

- Inhibition of PMN: leukotoxin
- Chemotaxis inhibitors
- Resistance to C' mediated killing
- Lymphocyte alterations: inhibit proliferation of T and B cells.
- Endotoxicity: strong, platelet aggregation, MØ killing
- Catalase: strong activity

4. Tissue Destruction:

- Enzymes: collagenase (some), alkaline phosphatase, acid phosphatase
- Toxic factors: epitheliotoxin, fibroblast growth inhibitors, endotoxins, LPS induced bone resorption

D. *Eikenella corrodens*: This is a small nonmotile, gram-negative, facultatively anaerobic rod.

1. **Disease Potential:** usually associated with polymicrobial infections but may also be the sole cause of infection. Outside of the oral cavity, *E. corrodens* can cause severe infections including septicemia, meningitis, brain abscess, and osteomyelitis. Often isolated together with *A.actinomycetemcomitans*.

Infections are usually the result of predisposing factors that compromise the body's host defense mechanisms and permit the organism to penetrate surrounding tissue.

E. *Fusobacterium nucleatum*: obligately anaerobic, Gram- negative, nonspore-forming rods

1. **Disease Potential:** long associated with fusospirochetal disease. Involved in acute orofacial abscesses. Outside of the oral cavity can cause a variety of diseases such as lung abscess and other pleuropulmonary infections.

F. *Capnocytophaga species*: fastidious, carbon dioxide requiring, Gram-negative, fusiform rods.

1. **Disease Potential:** isolated from a variety of clinical specimens in association with systemic disease in compromised hosts.

2. Colonization:

- Attachment: long fibers, capsule, vesicles, LPS
- Growth inhibitory factors: streptococci

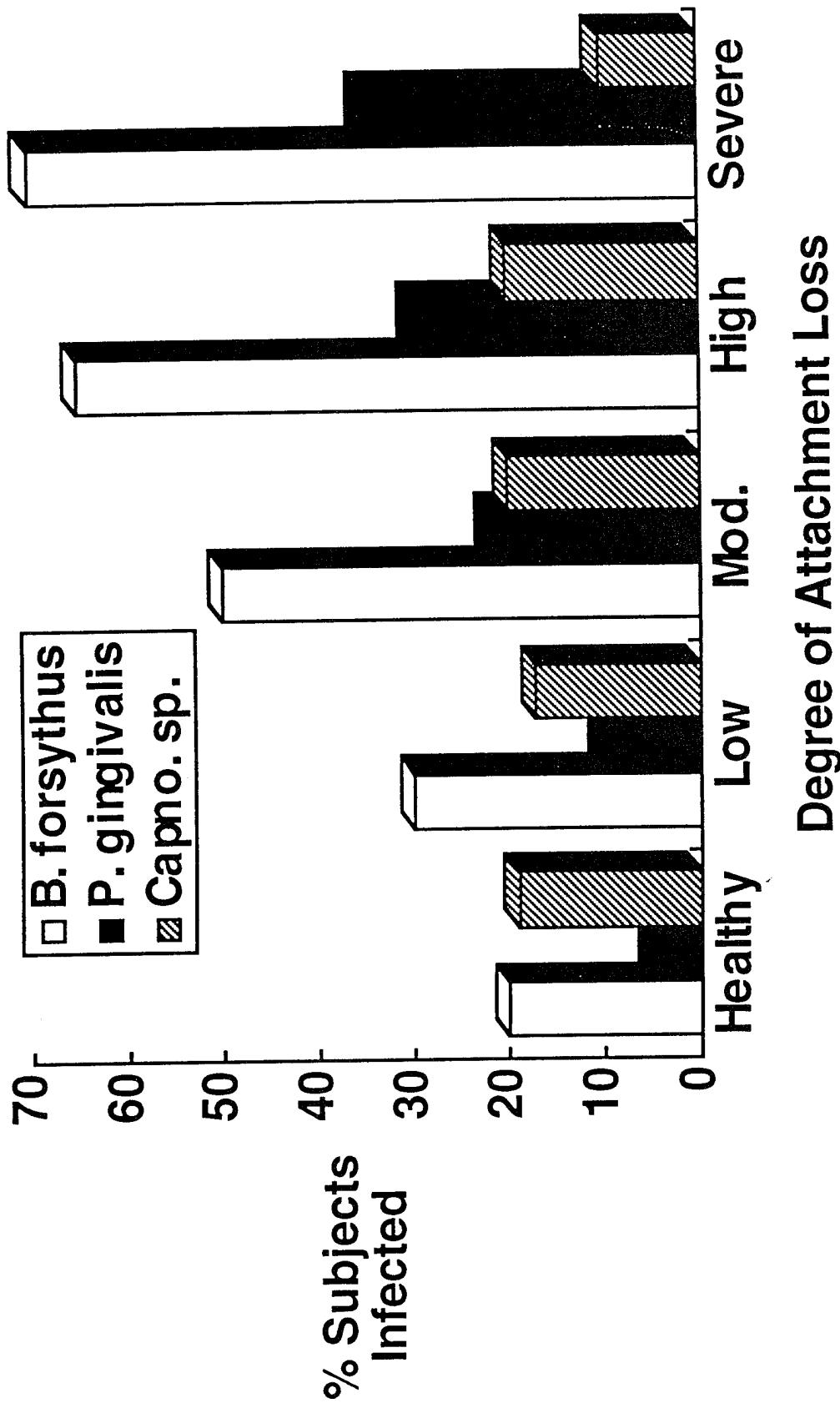
3. Evidence:

- Chemotaxis-inhibitors
- Lymphocyte alterations
- Endotoxicity
- Immunoglobulin proteases: IgA, IgG
- Superoxide dismutase

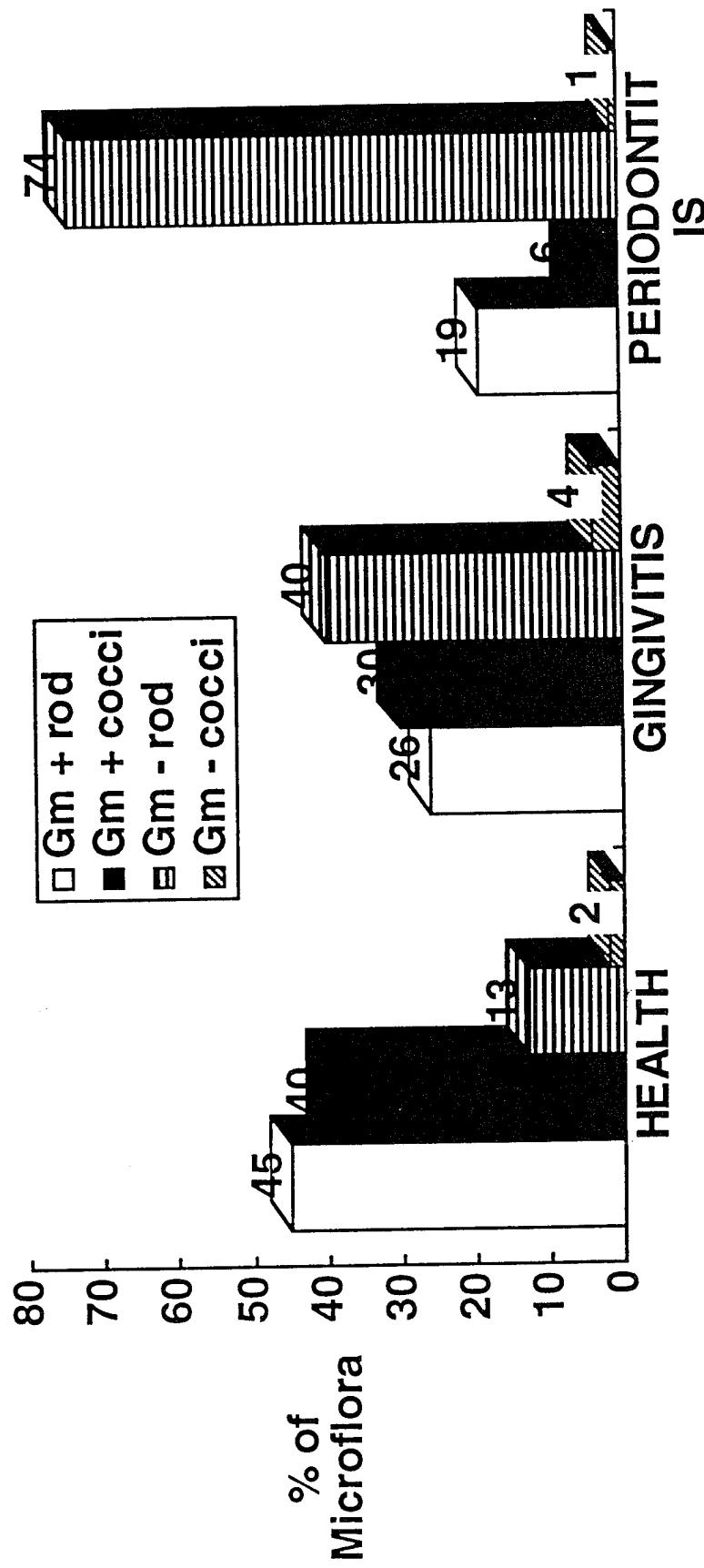
4. Tissue Destruction:

- Enzymes: trypsinase, aminopeptidase, alkaline phosphatase, acid phosphatase
- Toxic factors: epitheliotoxin, fibroblast growth inhibitors, endotoxin, LPS induced bone resorption

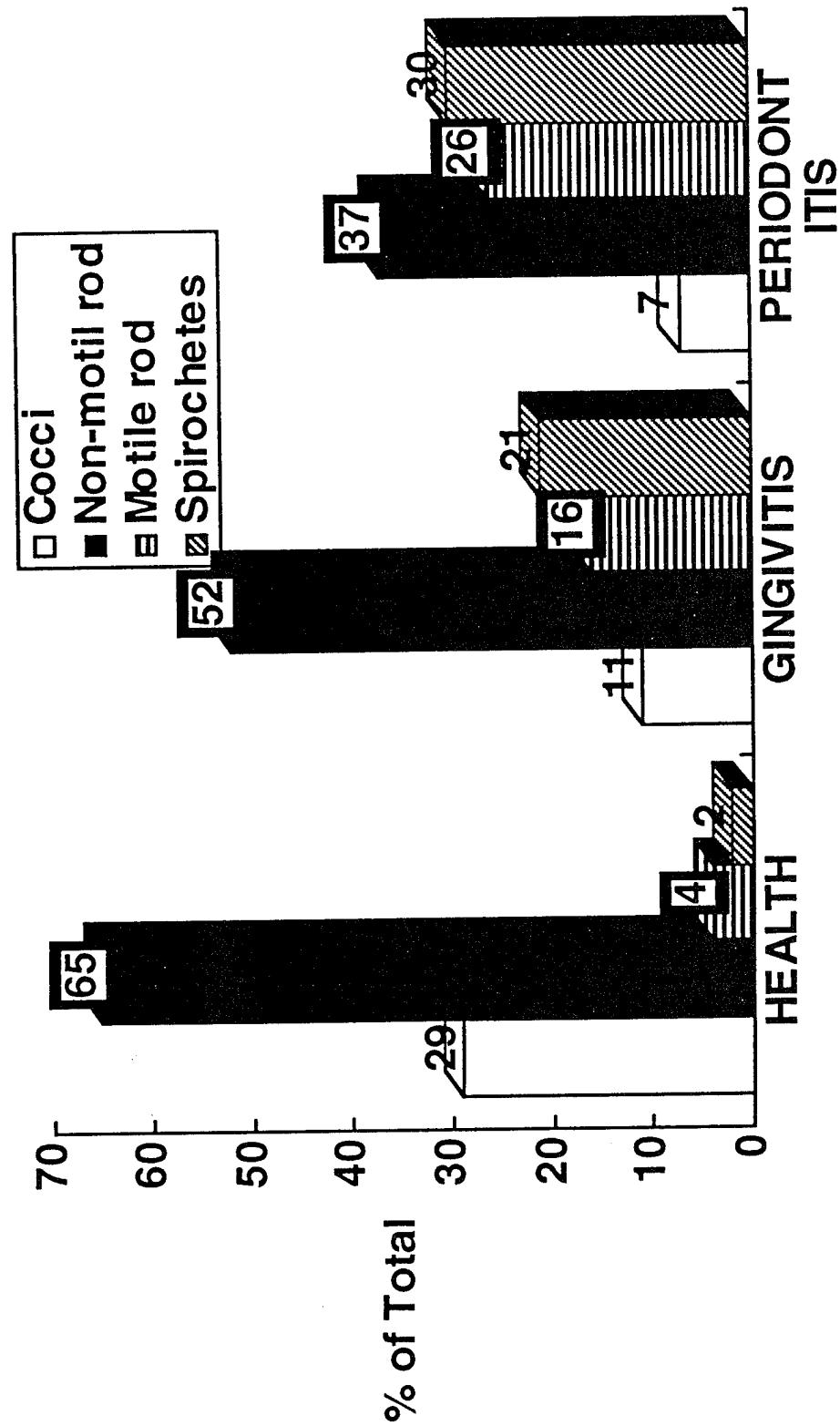
Infection and Attachment Loss



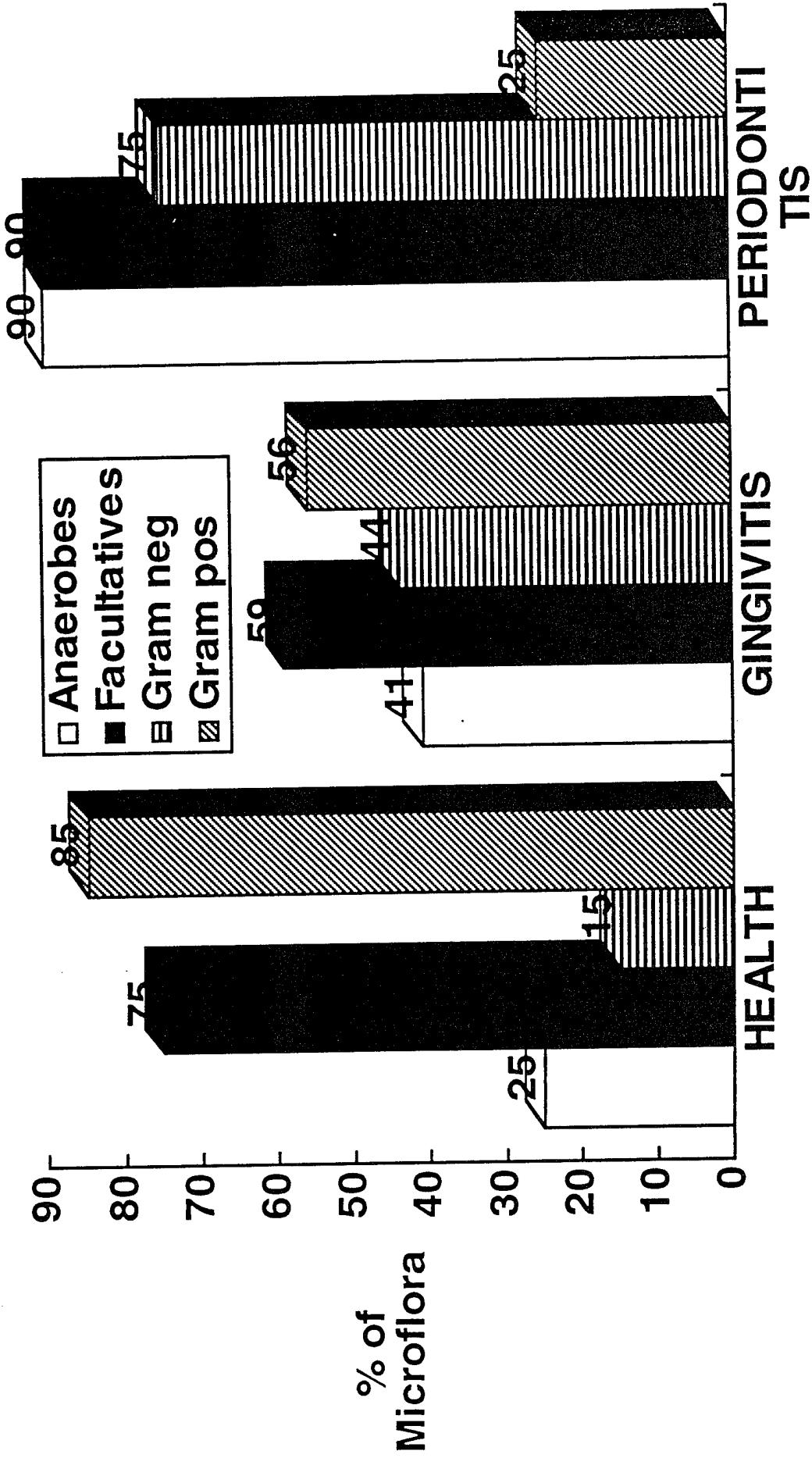
CULTIVABLE SUBGINGIVAL MICROFLORA



SUBGINGIVAL MORPHOTYPES



CULTIVABLE SUBGINGIVAL MICROFLORA



LECTURE 11: MICROBIOLOGY OF PULP AND PERIAPICAL INFECTIONS

Within the confines of the tooth as well as about its periapical regions are tissues and structures that are subject to infection. These tissues and the morphology of the structures they form are unique as is also the response of the region to infection. Localized infection of this region is common and can constitute a site from which infection spreads to other parts of the body. Successful management of endodontic diseases lies first in recognizing the role of microorganisms in the etiology of these diseases, followed by removal of the organisms and reestablishment of a sterile, intact pulp chamber sequestered from further microbial contamination.

I. Etiology Of Pulpal And Periapical Disturbances

A. Resistance Factors Of The Pulp Chamber: resistance factors including those similar to other parts of the body and those unique to the pulp-area are found.

1. **Systemic Factors:** include both specific and nonspecific components, such as macrophages, neutrophils, lymphocytes, complement, and all classes of immunoglobulins. These immune factors may be protective, but they also may mediate harmful reactions.

2. **Oral Factors:** protective elements of salivary, gingival, and mucosal secretions.

3. **Unique Factors:** enamel and dentin represent the first line of defense against external invasion of the pulp.

4. **Immunology:** The immunologic status of the pulp and periapical tissues is similar to that of other tissues. Little is known concerning the relative protective or injurious roles of the immune components in these diseased tissues, but there is no reason to believe that they are different from the rest of the body's tissues.

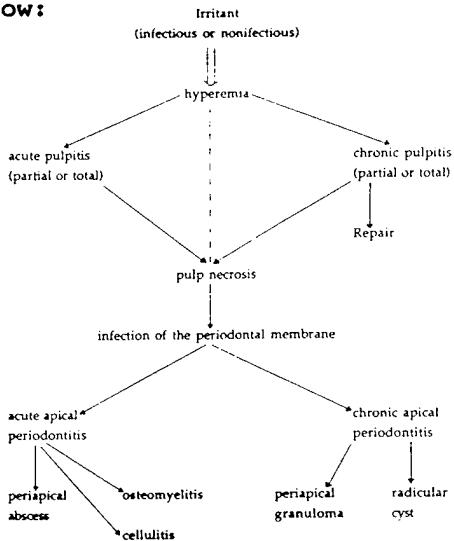
B. Inflammatory Reactions Of The Pulp: the pulp reacts rapidly with an inflammatory response (pulpitis) to various irritants, which can be microbially, thermically, mechanically, chemically, electrically, and irradiation induced. The consequence of the inflammatory reaction are somewhat unique because of the anatomical features of the pulp.

1. **Enclosure:** enclosure of the pulp tissue within hard dentin walls prevents its expansion during hyperemic and edematous phases of inflammation results in internal pressure.

2. **Tubules:** the tubules of the dentin allow chemicals and bacterial products to diffuse and influence the pulp even before microorganisms have reached the pulp chamber. In the early stages the odontoblastic layer may already be severely injured and destroyed.

3. **Repair:** the reparative capacity of the pulp is great. Pulp exposed to a variety of noninfectious injuries can heal under favorable conditions when the irritant is removed. Secondary reparative dentin is often produced, especially after physical trauma.

4. Infection: in infectious pulpitis, even before deep penetration of bacteria into the pulp tissue, acute involvement may result. This is especially true when the inflammatory exudate cannot escape. As a greater portion of the pulp becomes involved, the bacteria rapidly penetrate deeper, and the pain becomes more continuous and severe. The different pathways of pulpitis and apical periodontitis are shown below:



The character and extent of injury, the condition of the pulp tissue, the total bacterial load, and the virulence factors of the infecting microbes determine the course of bacterial infection. Virulence factors have either a direct influence on tissues or an indirect effect via inflammation.

C. Microbial Action on Pulp: Despite the natural shelter provided to the pulp, a few bacterial cells may penetrate it. Normally they are easily phagocytized and eliminated by the defense system of all healthy mesenchymal tissues. When the shelter is injured, the pulp can be infected.

1. Entrance pathways: the pulp tissues are enclosed in a chamber that is relatively impermeable to microbial invasion, but it is not resistant to entry by all microorganisms. An infection of the pulp may develop in several ways:

- a. through an open cavity caused by trauma such as fracture of the crown or root, operative dental procedures, or dental caries
- b. through the tubules of cut or carious dentin
- c. via the gingival crevice and by invasion along the periodontal ligament in several forms of periodontal disease
- d. by extension of periapical infection from adjacent infected teeth
- e. through the blood stream during bacteremia or septicemia. This process, called anachoresis, probably does not occur in healthy teeth, through which blood-borne bacteria pass unhindered.

D. Microorganisms Involved in Pulpal Infections Pulp infections share several characteristics with other common dental infections such as caries and periodontal diseases. The first is that they usually are caused by indigenous organisms. Second, the pathogens are of relatively low virulence. Finally, no single bacterium has been identified as the single cause of the disease. Infections average 5 or 6 taxa and can range as high as 13. Streptococci are most prevalent.

1. Microbial Virulence Factors: Although microbes usually involved in pulp infections are of relatively low virulence, several potential virulence factors have been associated with them. Gram-negative bacteria containing endotoxin are common in pulp and periapical infections. In addition, teichoic acids and peptidoglycans, which possess endotoxin-like activity, also are products of the bacteria in pulp infections. Numerous bacterial enzymes are suspected of being involved in pathologic processes. *Bacteroides* produce collagenase, hyaluronidase, fibrinolysin, and alkaline and acid phosphatases. Metabolic end products (ammonia, hydrogen sulfide, toxic amines, organic acids) that may be injurious to pulp and periapical tissues are produced by many of the bacteria associated with endodontic lesions. Bacterial antigens also can stimulate immune responses that lead to hypersensitivity reactions.

2. Nature Of The Pulp: an open pulp chamber would enhance the growth of aerobic oral bacteria. A closed pulp chamber would enhance the growth of anaerobes. A vital tooth would contain primarily aerobic organisms whereas nonvital teeth are frequently infected with obligate anaerobes.

3. Organisms Of Special Significance: although pulp infections are non-specific, *Bacteroides* seem to be associated with pain and destruction of tissue in endodontic lesions.

Microorganisms Isolated From Infected Root Canals

Aerobic or Facultative Microorganisms	Anaerobic Microorganisms
<i>α</i> -Hemolytic streptococci	<i>Lactobacillus</i> species
Nonhemolytic streptococci	<i>Bacteroides</i> species
Enterococci	<i>Peptococcus</i> species
Group D streptococci	<i>Peptostreptococcus anaerobius</i>
<i>Corynebacterium</i> species	<i>Bifidobacterium</i> species
<i>Bacillus</i> species	<i>Eubacterium</i> species
<i>Staphylococcus epidermidis</i>	<i>Propionibacterium acnes</i>
<i>Proteus</i> species	<i>Veillonella</i> species
<i>Escherichia coli</i>	<i>Actinomyces</i> species
<i>Candida albicans</i>	<i>Porphyromonas</i>

E. Bacterial Ecology of the Infected Root Canal: the microbial composition of an infected root canal is determined by:

- the route by which the bacteria gain access to the root canal
- the number and quality of ecological factors

The infecting bacteria of the root canal are in a dynamic state, influenced by:

1. Interactions between bacteria: although the root canal flora is much less complex than the subgingival flora, a similar kind of ecological relationship exists between the bacteria.

2. Bacterial interactions with host factors:

F. The Periapical Reaction: When the pulp becomes necrotic, bacteria advance through the total necrotic tissue of the root canal, and an inflammatory reaction in the periapical tissues (**apical periodontitis**) develops.

1. **Microbiology:** the few studies of the microbial flora of periapical infections indicate that the bacteria present in these lesions are very similar to those found in intact, necrotic teeth. Strict anaerobes predominate, and the species present resemble those found in subgingival plaque associated with periodontal diseases. Periapical infections are dominated by obligately anaerobic bacteria. *Prevotella*, *Prophyromonas*, *Fusobacterium*, and *Peptostreptococcus* are common and combinations of groups seem to play a more essential role than others. On the other hand, *Prevotella* and *Porphyromonas* species by themselves do not possess the capacity to survive and cause reactions.

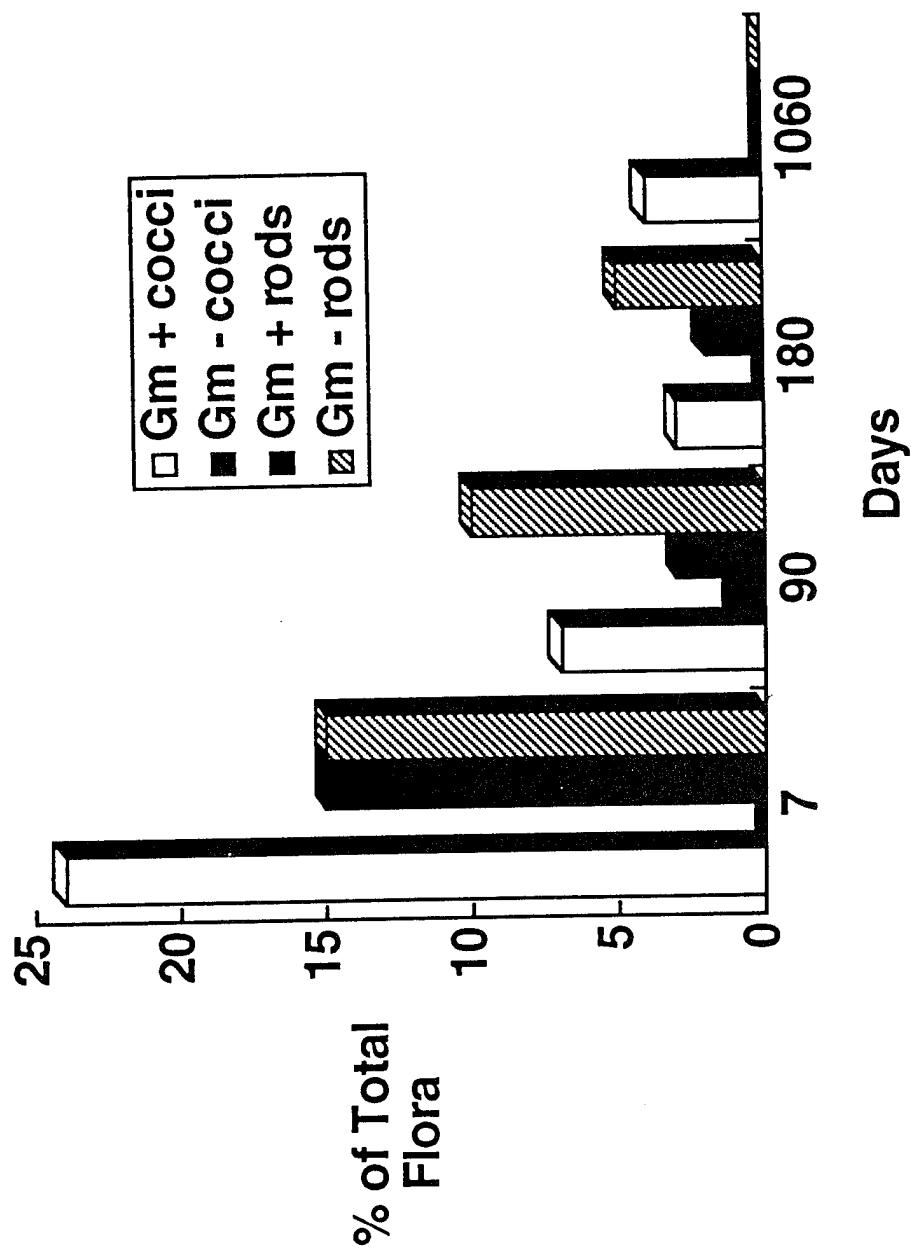
G. Endodontic Culturing: the purpose of endodontic therapy has been described as the restoration of the involved tooth to its proper form and function in a healthy state. If it is to be healthy, it must be free of microbes, and the most practical method of determining if the bacteria have been destroyed or removed by instrumentation and medications is by bacteriologic culturing. Proponents of routine culturing admit that it is a less than perfect method but believe that it is much better than doing nothing. However:

- a. several investigators have reported that bacteria are frequently present in root canals that display negative cultures;
- b. the techniques used are subject to contamination from oral flora, resulting in misleading interpretations of the actual conditions within the chamber.

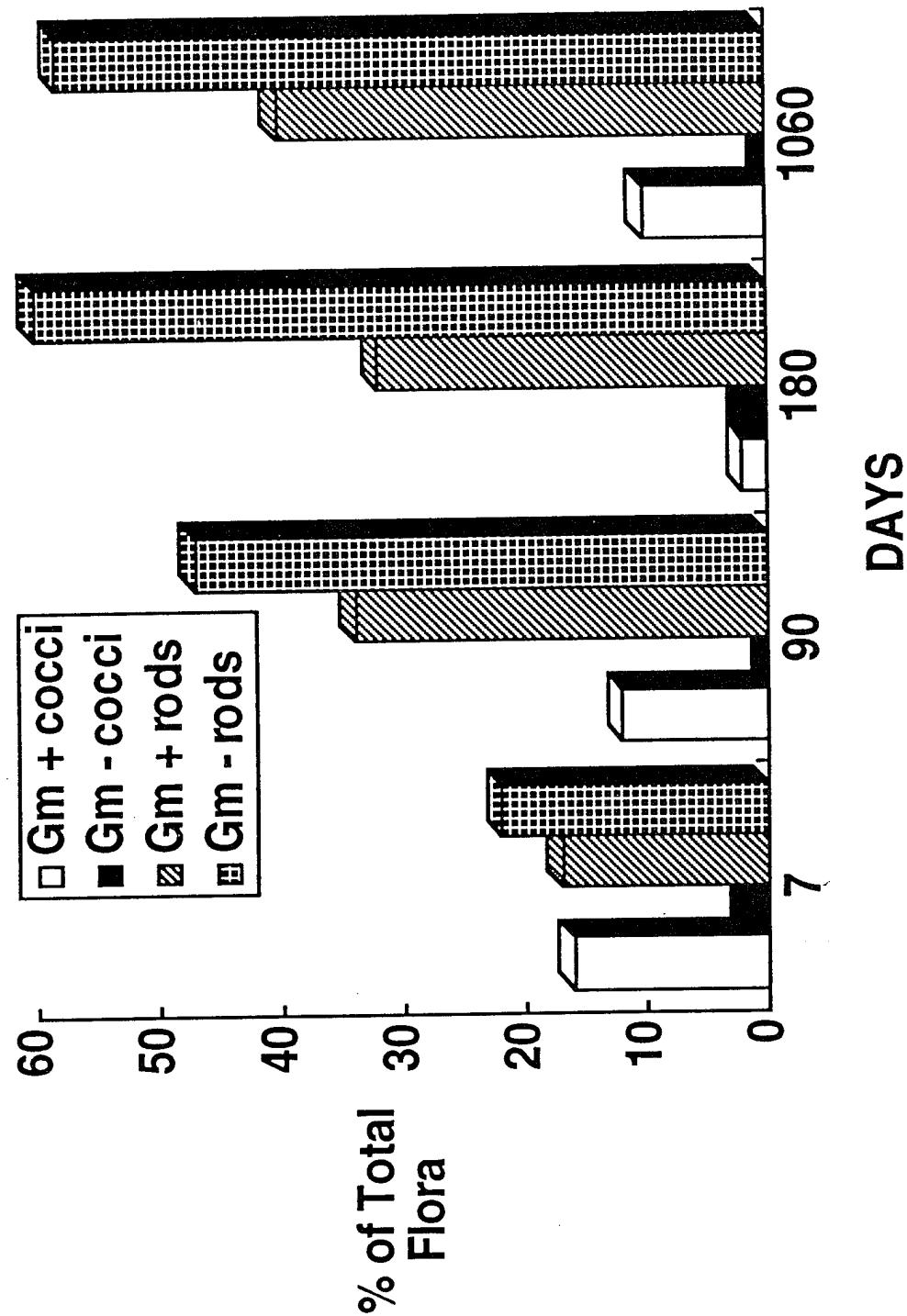
COMMONLY ISOLATED BACATERIA FROM NECROTIC INFECTED PULPS

	Genus	Common species/group
Obligately anaerobic bacteria		
Gram-negative rods	<i>Porphyromonas</i> <i>Prevotella</i> <i>Mitsuokella</i> <i>Fusobacterium</i> <i>Selenomonas</i> <i>Campylobacter</i> <i>Wolinella</i> <i>Treponema</i>	<i>P.gingivalis</i> , <i>P. endodontalis</i> , <i>P.oralis</i> , <i>P. oris</i> , <i>P.buccae</i> , <i>P. intermedia</i> , <i>P.melaninogenica</i> <i>F.nucleatum</i> , <i>F.necrophorum</i> <i>S.sputigena</i> <i>C.sputhorum</i> <i>W.recta</i>
Gram-positive rods	<i>Eubacterium</i> <i>Propionibacterium</i> <i>Arachnia</i> <i>Lactobacillus</i> <i>Actinomyces</i>	<i>E.alactolyticum</i> , <i>E.lentum</i> <i>P.acnes</i> <i>A.propionica</i> <i>L.catenaforme</i> , etc. <i>A.naeslundii</i> , etc.
Gram-positive cocci	<i>Peptostreptococcus</i>	<i>P.anaerobius</i> , <i>P.micros</i> , <i>P.prevotii</i> , <i>P. asaccharolyticus</i> , <i>P.magnus</i>
Gram-negative cocci	<i>Veillonella</i>	<i>V.parvula</i>
Facultatively anaerobic bacteria		
Gram-positive cocci	<i>Streptococci</i> <i>Enterococcus</i>	<i>S.mititis</i> , <i>S.anginosus</i> , <i>S.oralis</i> , <i>S.intermedius</i> <i>E.faecalis</i> , <i>E.faecium</i>
Gram-negative rods	<i>Eikenella</i> <i>Capnocytophaga</i>	<i>E.corrodens</i>
Gram-negative cocci	<i>Neisseria</i>	
Gram-positive rods	<i>Corynebacterium</i> <i>Lactobacillus</i>	<i>C.xerosis</i>

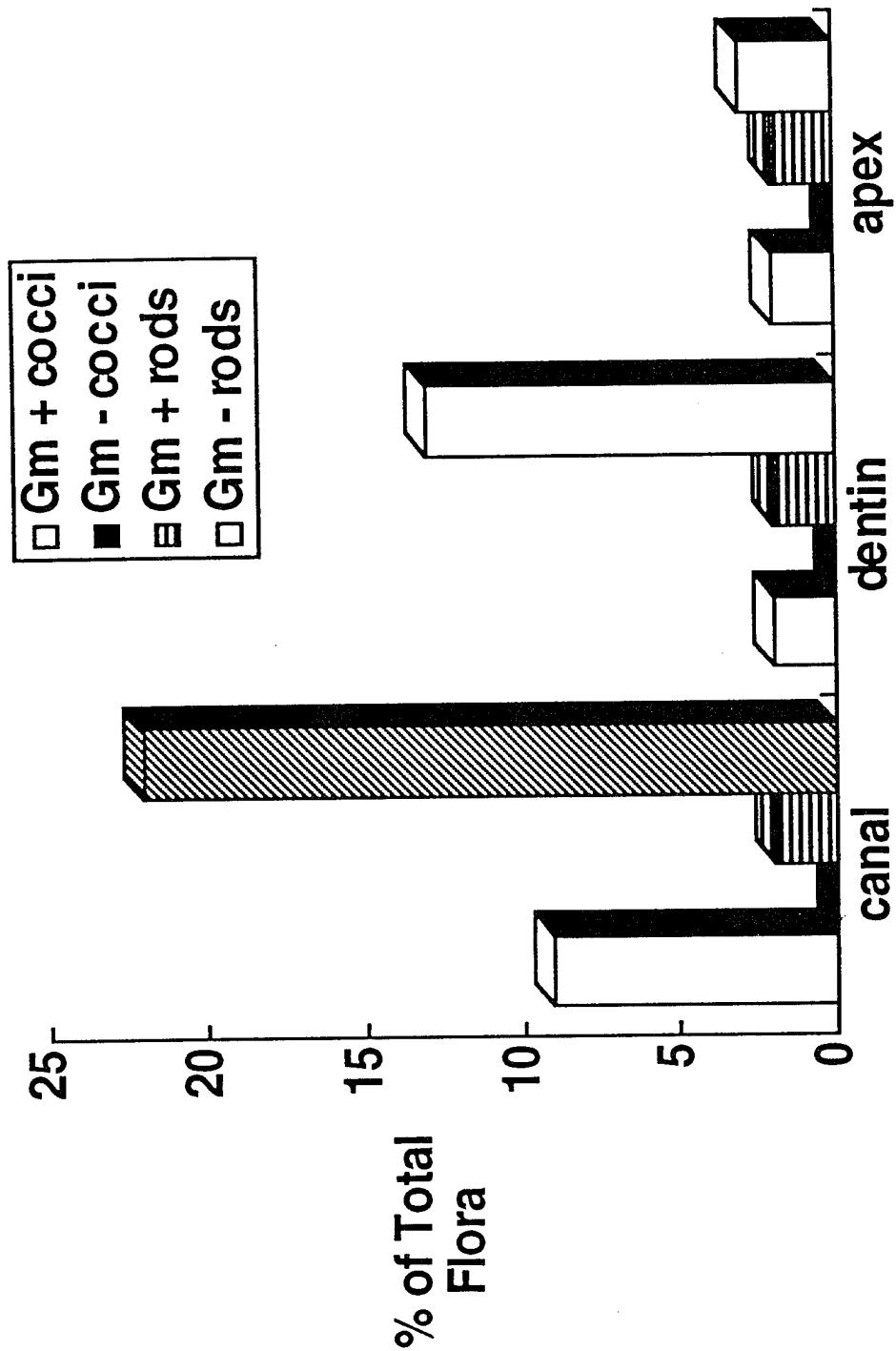
Facultatively Anaerobic Bacteria



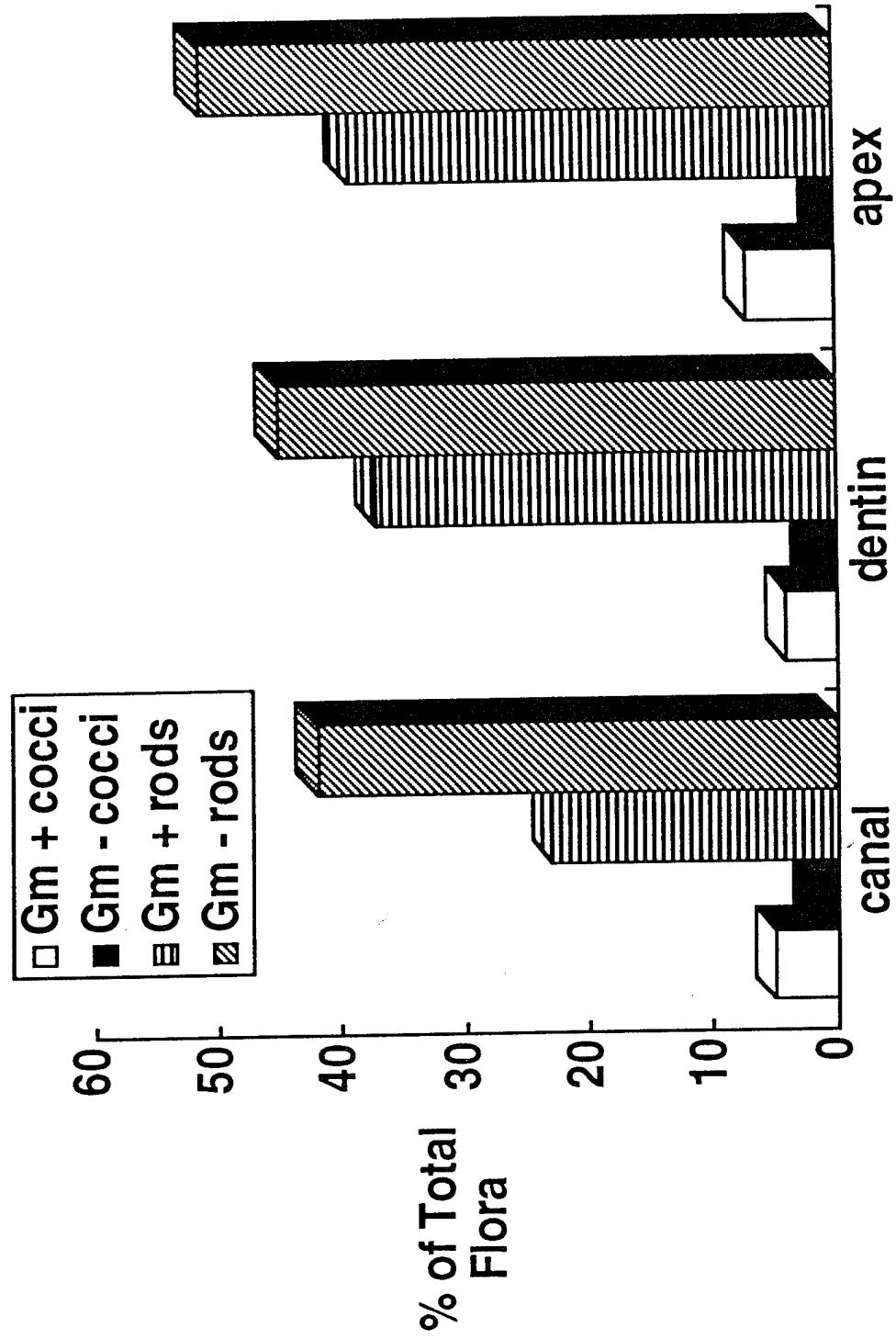
Obligately Anaerobic Bacteria



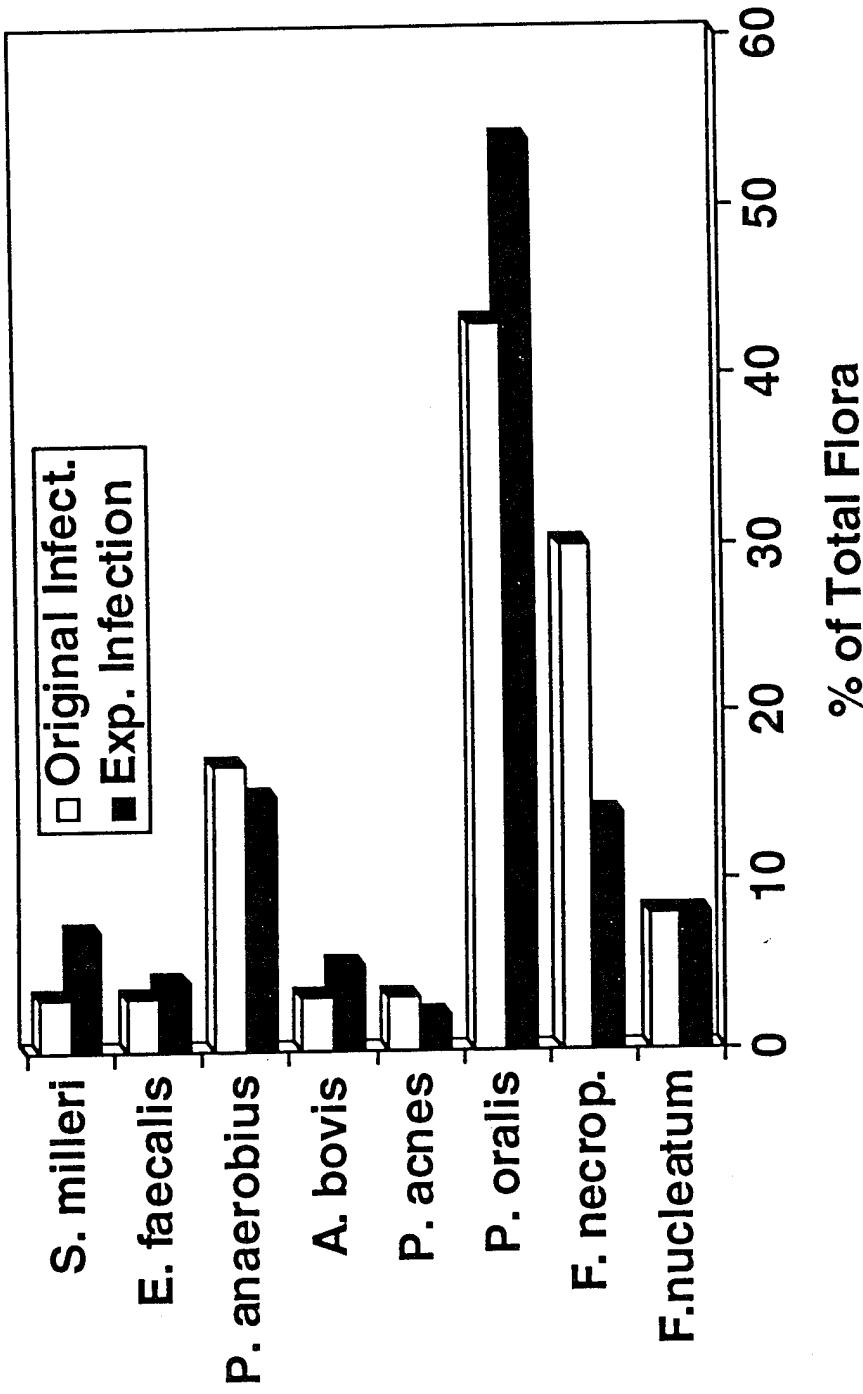
Facultatively Anaerobic Bacteria



Obligately Anaerobic Bacteria



Bacterial Succession



LECTURE 12: PATHOGENIC POTENTIAL OF THE NORMAL ORAL FLORA

The primary pathogenic potential of the normal oral flora is the causation of periodontal diseases, caries, and abscesses. With the exception of these diseases, members of the normal oral flora are traditionally considered nonpathogenic. However, this is only true as long as they are confined within the mouth. Once oral microorganisms gain access to the bloodstream, they can establish in various parts of the body and cause local damage. If vital organs are involved, this may have serious consequences. Despite advances in antimicrobial therapy, the morbidity and mortality of systemic infections caused by oral microorganisms has remained significant. Oral bacteria can enter the blood stream as a result of translocation of an organism from one part of the body to another, a weakening of the body's immune system, or a shift in microbial ecology. Certain oral bacteria are more prone to cause systemic infections than others, and some show a curious predilection for particular tissues of the body. Several species often work in concert, especially when causing abscesses. In such mixed infections, anaerobic bacteria, like *Bacteroides melaninogenicus* are usually involved.

I. Dissemination of oral bacteria in the body: dissemination of oral bacteria by means by the bloodstream is not a rare event. Less than one minute after the extraction of a tooth or surgery on gingival tissues, it is almost invariably possible to cultivate oral bacteria from, for example, the cubital vein. This means that within this short time period, bacteria have spread from the mouth, have passed through the heart and lungs, and have made their way through the peripheral capillary system. Certain microorganisms that enter the bloodstream from the oral cavity can cause oral infections through a process known as *anachoresis*. In addition to surgical procedures, bacteria may enter the bloodstream from deep carious lesions, the infected periodontal pocket and by way of a fractured tooth.

A. Role of Host Immunity: the body's immune system is weakened when treated with immunosuppressants, which occurs in certain cancers and transplant surgery. Under these conditions, normal flora may be able to cause disease. Oral organisms, such as *C.albicans*, need to be controlled to prevent serious infections. The balance within the oral cavity can be tilted to disease by poor oral hygiene or other systemic diseases that decreases local resistance.

B. Changes in Oral Microbial Ecology: many factors interact to keep oral flora in check. Should major changes occur then it is possible for endogenous normal flora to get out of control. The ecological balance within the oral cavity can be tilted to disease by poor oral hygiene leading to changes in antagonistic factors in the oral ecosystem. Changes in the flow of saliva is one important factor.

II. Oral And Systemic Disease Associated With Oral Bacteria: Oral infections may occasionally act as foci for disease or injury at other body sites. That dental focal infections (infections that have as their port of entry the oral cavity) may induce metastatic systemic effects was emphasized during the early twentieth century but has been ignored for many years. However, recent progress in bacterial classification and the realization that certain organisms are normally found only in the oral cavity have opened the way for a more accurate assessment of the risk of dental focal infection. It is increasingly clear that the oral cavity can act, especially in compromised patients, as the origin for dissemination of pathogenic organisms to distant body sites.

A few examples of systemic disease stemming from either oral disease or from specific oral flora are shown in the following table:

Oral and Systemic Disease Associated with Oral Bacteria

Genus	Oral Disease	Systemic Disease
<i>Lactobacillus</i>	Dental Caries	Pleuropulmonary infections, Cystitis
<i>Fusobacterium</i>	Ulcerative gingivitis	Human bites, bacteremia, upper respiratory infections
<i>Actinobacillus</i>	Juvenile periodontitis	Infective endocarditis, actinomycosis
<i>Streptococcus</i>	Dental caries, abscesses	Infective endocarditis
<i>Eubacterium</i>	Periodontitis, pulpitis	None
<i>Peptostreptococcus</i>	Periodontitis, pulpitis	Pulmonary abscess, brain abscess
<i>Haemophilus</i>	Acute bacterial epiglottitis	Pneumonia, sinusitis, meningitis
<i>Eikenella</i>	Periodontal disease	Soft tissue infections
<i>Wolinella</i>	Dental abscesses	Abdominal infections
<i>Capnocytophaga</i>	Juvenile periodontitis	Pneumonia, infective endocarditis

I. Mechanisms of secondary infection:

- metastatic spread of infection from the oral cavity as a result of transient bacteremia
- metastatic injury from the effects of circulating oral microbial toxins, and
- metastatic inflammation caused by immunological injury induced by oral microorganisms

II. Types of Infections

A. Brain Infections: the incidence of brain abscesses caused by dental foci is thought to be low, but the case fatality rate among reported cases is relatively high. Multiple brain abscesses have been seen from infection with *S.mutans*, *S.millerii*, *P.melaninogenica*, *Peptostreptococcus*, and *Fusobacterium nucleatum*. Cerebral infarction has also recently been related to dental infection. LPS of gram-negative oral bacteria introduced into the bloodstream from recurrent transient bacteremias are postulated to predispose for cerebral infarction by damaging vascular walls and promoting thrombi formation.

B. Neurological Disorders: Chronic meningitis has been related in some cases to periapical abscesses and dental caries on maxillary teeth. Acute meningitis secondary to systemic antibiotic treatment of periodontitis has been described. Tetanus resulting from tooth extraction has also been described.

C. Head and Neck Infections: Chronic maxillary sinusitis, Ludwig's angina, and facial plane infections may result from spread of periodontal and periapical infections into tissues immediately surrounding the oral cavity. Spread of orofacial infections usually follows pathways created anatomically by fascial planes and spaces. Ascending orificial infections may reach the brain, cavernous sinus, and orbit while descending orificial infections may involve the mediastinum.

D. Heart Infections and Disorders: Subacute infective endocarditis is commonly caused by oral bacteria introduced into the bloodstream via oral hygiene procedures or trauma from dental therapies. Most cases are attributed to viridans streptococci, particularly *S.sanguis* and *S.mutans*. *A.actinomycetemcomitans* is increasingly implicated in cases of infective endocarditis.

Infective Endocarditis is a disease resulting from microbial colonization of a focal area of the endothelial membrane covering the inner surfaces of the heart and its valves. The course of the disease is described as either acute or subacute infective endocarditis. When caused by oral bacteria, the disease usually takes the subacute form. This form begins insidiously and pursues a long clinical course lasting several months.

1. Predisposing conditions: in contrast to the acute form, subacute infective endocarditis usually affects heart valves, the surfaces of which have already been damaged. The classical cause of such predisposing alterations is post-streptococcal rheumatic fever. However, in recent years, additional risk groups have emerged. These include survivors of cardiac surgery, narcotic abusers, and patients with a generally low resistance to infection.

2. Etiology: the streptococci found in the mouth, often referred to as "viridans streptococci". remain the most important oral bacteria responsible for infective subacute endocarditis. However, Staphylococcal endocarditis is especially prevalent in drug addicts or as a postoperative complication where blood can become contaminated with the microflora of the skin. Recent studies indicate that the microorganisms most often causing endocarditis exhibit a high degree of adherence to human heart valves, or monolayers of explanted endothelial tissue.

E. Lung and Thoracic Infections: Lung abscesses may be caused by the aspiration of salivary or dental plaque bacteria or septic emboli induced by odontogenic infections. A third of pulmonary abscesses have been attributed to oral foci. Chronic obstructive pulmonary infections in cystic fibrosis patients are frequently caused by mucoid variant strains of *Pseudomonas aeruginosa*, which colonize the tongue and buccal mucosa and are aspirated into the lungs.

F. Hematological infections: Septicemia in acute leukemia and immunocompromised patients may result from *Enterobacteriaceae* species, pseudomonads, staphylococci, and other organisms originating from the oral cavity. These species may seed into the bloodstream from infected periodontal pockets and mucosal lesions. In patients with sickle cell anemia, periodontal abscesses and pericoronitis may precipitate a life-threatening sickle cell crisis. Prosthetic joint infections involving oral *Peptostreptococcus micros* and viridans streptococci can occur from hematogenous spread induced by infection or restorative dental care. In addition, toxic shock syndrome may develop from *Staphylococcus aureus*-associated odontogenic infections.

G. Eye Infections: Endogenous endophthalmitis has been caused by *A.actinomycetemcomitans* or oral origin. Orbital cellulitis from oral microorganisms has also been shown to occur after tooth extractions and periapical abscess formation. Inflammation of the uveal tract of the eye also has been related to pulp and periodontal disease.

H. Skin Infections and Diseases: Skin infections may occur after traumatic inoculation of microorganisms from the oral cavity. Human bites, clenched-fist injury, and puncture wounds are commonly infected by a variety of oral bacteria. Chronic urticaria, has also been caused by oral microflora.

I. Abscess Formation in Internal Organs: In addition to possible emboli derived from cardiac vegetation, abscesses in internal organs are occasionally caused by oral bacteria as a result of direct or indirect dissemination from the oral cavity. *Streptococcus milleri*, which is relatively rare in endocarditis, is one of the most frequent isolates from purulent abscesses of the brain, liver, and other internal organs. Oral anaerobic bacteria, like fusobacteria, anaerobic streptococci, and actinomyces are also among the dominant causes of brain abscesses.

J. Aspiration Pneumonia: Aspiration of oropharyngeal secretions and excised oral tissue into the lungs are common events that can initiate pulmonary infection. The important role of oral bacteria in pulmonary disease is well-documented. Oral bacteria are isolated with high frequency, and this type of disease is often correlated with severe gingivitis and large deposits of dental plaque. Altered consciousness due to alcoholism, general anaesthesia, or the use of drugs which depress the cough reflex are conditions that increase susceptibility to aspiration pneumonia. Aspiration of tooth fragments during tooth extraction is not uncommon. The bacteria most frequently implicated are bacteroides, fusobacteria, and anaerobic and microaerophilic streptococci. *B.melaninogenicus* often seems to play a key role.

Final Comment: The dental profession should be aware of the systemic hazards of dental focal infections. Prompt recognition and treatment of dental foci are essential to minimize secondary morbidity and mortality in high-risk patient groups. Immunocompromised patients, in particular, warrant thorough dental evaluation to prevent the life-threatening effects of dental focal infections. The incidence of dental focal infections in systemically healthy and in compromised individuals needs to be established, but it is probably low.

Significance and main occurrence of microbes in samples from subepithelial infections and their relation to frequency and pathogenicity

Microorganisms	Normal	Significance in subepithelial infections		Pathogenicity
		Frequency	Significance	
Gram-positive cocci				
<i>Staphylococcus aureus</i>	+	(+)	++	+++ (+)
<i>Staphylococcus epidermidis</i>	+	(+)	+	(+)
Other micrococci	+++	+	+	+
<i>Streptococci (viridans)</i>	-	++	++	++
<i>Peptostreptococci</i>				
Gram-negative cocci				
<i>Neisseria</i>	++	+	-	
<i>Veillonella</i>	+	+	+	
Gram-positive rods				
<i>Corynebacterium</i>	+	+	-	
<i>Lactobacillus</i>	+	++	-	
<i>Propionibacterium</i>	(+)	+	++	++
<i>Eubacterium</i>	+	++	++	++
<i>Actinomyces israelii</i>	+	++	++	++
Other <i>Actinomycetes</i>	+	++	+	++
<i>Nocardia</i>	+	+	+	++
Gliding or corroding bacteria				
<i>Capnocytophaga</i>	(+)	+	+	
<i>Wolinella</i>	-	+	++	
<i>Eikenella</i>	(+)	+	++	
Gram-negative anaerobic rods				
<i>Porphyromonas gingivalis</i>	-	+++	+++	+++
<i>Prevotella intermedia</i>	(+)	+++	+++	+++
<i>Prevotella melaninogenica</i>	(+)	++	++	++
Nonpigmented <i>Prevotella</i>	(+)	?	?	?
<i>Fusobacterium</i>	-			
<i>Treponema</i>				
<i>Haemophilus</i> (excluding <i>H. influenzae</i>)	++	(+)	(+)	(+)
<i>Actinobacillus</i>	(+)	+	+	+
<i>actinomycetemcomitans</i>		(-)	(-)	+++
Enteric bacteria				

LECTURE 13: MICROBIAL GENETICS AND GENETIC ENGINEERING

Bacteria are able to respond rapidly to changes in the environment. They do so by regulating their metabolism so that metabolic activities are expressed only when needed. The primary form of regulation is control of expression of genes. Bacteria have access to a variety of ways to introduce genetic variation into populations of cells. When the population faces a new form of selective pressure, a few cells may have an altered gene, the altered function of which will allow those cells to survive. These altered genes can spread throughout a population by rapid growth and by genetic exchange. This has profound implications when we consider treatment of infectious disease in dental offices, clinics, and hospitals.

The revolution in genetic engineering also has dental significance. The genetic engineering approach is valuable in understanding how pathogens, such as *S. mutans*, causes oral disease. The value of genetic engineering to the dental professions already is apparent in the release of a new genetically engineering hepatitis B vaccine.

I. Organization Of Genes: the genetic material of a typical bacterium consists of a single circular DNA molecule with sufficient genetic information to code for about 2000 proteins with an average molecular weight of 50,000.

A. **DNA:** the cellular constituent that contains information for controlling activities of a cell during the present generation and for specifying the inheritance of the next generation. DNA consists of deoxyribose, phosphate, the purines adenine and guanine, and the pyrimidines thymidine and cytosine. Most DNA is double stranded with adenine binding to thymidine and cytosine binding to guanine.

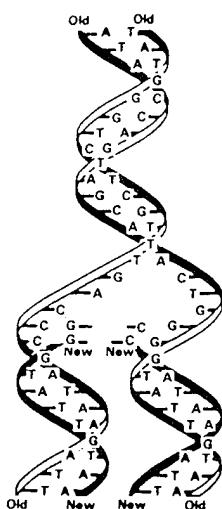
B. **RNA:** the function of DNA is mediated by a second type of nuclei acid, ribonucleic acid or RNA. RNA differs from DNA in that it is single stranded, contains ribonucleotides rather than deoxyribonucleotides, and consequently uridine in place of thymidine. There are three classes of RNA, all of which are involved in expression of the information stored in DNA. They are messenger RNA (mRNA), which codes for enzymes and other proteins, ribosomal RNA (rRNA), a structural component of ribosomes, and transfer RNA (tRNA), which is used in the transfer of amino acids to growing polypeptides during protein synthesis. The three types of RNA are transcribed from DNA.

C. **The Eukaryotic Genome:** composed of numerous paired chromosomes (diploid) each made up a fiber of chromatin containing histone and non-histone proteins. Eukaryotic DNA is divided into areas called exons and introns. Exons are the sequences of DNA that are translated into gene products and introns are intervening sequences that are not translated into gene products.

D. **The Prokaryotic Genome:** composed of a single circular molecule of DNA. Bacterial genes are haploid and bacterial DNA do not contain introns.

E. **The Viral Genome:** many contain double stranded or single stranded DNA or single stranded RNA.

II. Replication: double-stranded DNA is synthesized by semiconservative replication.



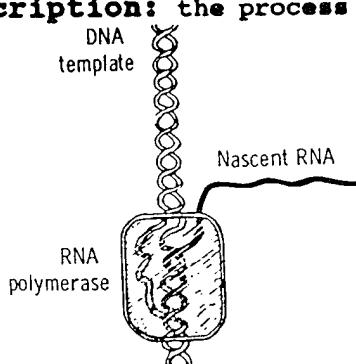
As the parental duplex unwinds, each strand serves as a template for DNA replication. New strands are synthesized with their bases in an order complementary to that in the preexisting strands. When synthesis is complete, each daughter molecule contains one parental strand and one newly synthesized strand.

A. Eukaryotic DNA: replication begins at several growing points along the linear chromosome. Cells have evolved special machinery known as the mitotic spindle, that pulls daughter chromosomes into separate nuclei newly formed by the process of mitosis. Meiosis halves the chromosomal number of diploid cells to form haploid cells called gametes, which fuses to form zygotes. These serve as the primary source of genetic variability via recombination.

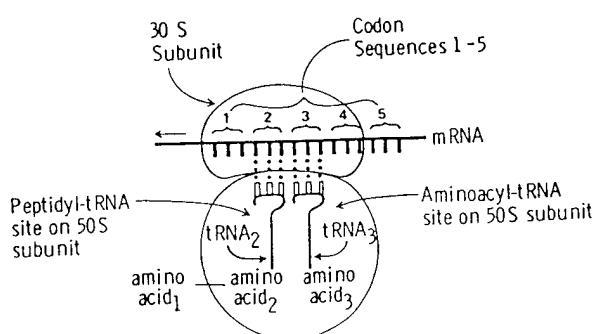
B. Bacterial DNA: lack anything resembling the complex structures of eukaryotes. Prokaryotic replicons are believed to be linked to the cell membrane, and segregation of daughter chromosomes and plasmids is thought to be coupled to elongation and septation of the membrane.

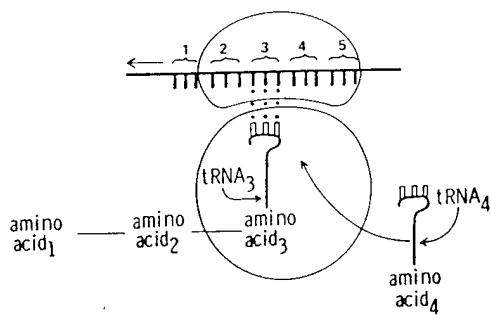
III. Expression Of Information Stored In DNA: the information contained in the sequence of purine and pyrimidine bases in DNA is used to carry out and control cellular activities.

A. Transcription: the process in which information contained in DNA is copied to make RNA containing the same information. Transcription is mediated by RNA polymerase, which copies the information from one of the strands of DNA.



B. Translation: the final step in the expression of information stored in DNA is the translation of that information from the sequence of adenines, guanines, cytosines, and uracils of mRNA to the sequence of amino acids in proteins. The amino acid sequence of protein is determined by the base sequence

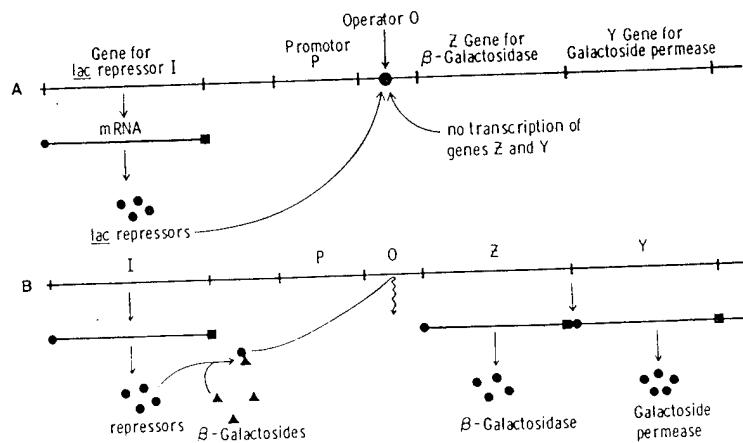




of an mRNA. The mRNA sequence is subdivided into units called codons, and each codon specifies one amino acid. A codon uses a sequence of three bases to code for one amino acid; for example, UUU codes for phenylalanine. Since there are four bases in mRNA and each codon uses three bases, there are 64 possible codons.

IV. Control Of Expression Of Genetic Information: All activities of living cells are orchestrated into a harmonious biological symphony by a network of regulatory circuits designed to maximize the return to the cell for its metabolic investment in terms of energy and building blocks. Coarse regulation is at the gene level while fine regulation occurs at the catalytic level.

A. Transcriptional Control: the expression of genes in prokaryotes may be regulated in groups or independently. Each unit of transcription is called an **operon**, a cluster of contiguous related genes all controlled by their own set of regulatory elements. As an example we will consider the control of lactose catabolism. Here, some genes code for regulatory proteins and others code for enzymes. Consider the regulatory function carried out by a class of proteins called **repressors**. The function of repressors is to control the transcription of genes, coding for various enzymes. The repressor for enzymes of lactose catabolism is called the lac repressor. The lac repressor is encoded in a gene I separate from the other genes of lactose catabolism. The lac repressor can recognize and bind to a specific DNA base sequence, called the **operator** or O. The operator is part of a larger sequence known as the **promoter**. The promoter is the site for binding of RNA and this can be regarded as the place where transcription is initiated. The lac repressor normally is active and binds to the lac operator, blocking transcription. In this way, bacteria growing in the absence of lactose are prevented from making galactoside permease, which transports lactose into the cell, and β -galactosidase (product of the lac Z gene), which cleaves it into glucose and galactose. When these same bacteria are grown on lactose rather than, for example, glucose, a few lactose molecules will get onto the cell by means of diffusion. These lactose molecules will bind to the lac repressor at a site distinct from the operator-binding region. The lac repressor is an allosteric protein, which changes conformation on binding lactose. A consequence of this conformational change is that the repressor can no longer bind to the operator region. The inactivated lac repressor detaches from DNA. RNA polymerase then can bind to the promoter and initiate transcription, beginning expression of the genes involved in lactose catabolism.



V. Mutation And Gene Rearrangement

A. **Mutation:** an abrupt change in structure of a gene is called a mutation, and organisms possessing the change are mutants. The mutation may cause the gene or its product to work better, poorer, or not at all and are produced by a physical or chemical change in the structure of a gene.

B. **Recombination:** recombination is a process by which new combinations of genes are formed in an organism. Recombination in bacteria occurs after fragments of DNA are transferred from a donor cell to a recipient and involve a gene called *rec*. In recent years a *rec-independent* type of recombination has become important because of the problems it has given in the treatment of infectious diseases with antibiotics. The recombination process is called *transposition* after the ability of antibiotic-resistant genes to transpose or move from one site to another within a single chromosome. The genes are carried on DNA fragments called *transposons*, which can insert into other DNA molecules. *Transposons* carry genetic information coding for the enzymes needed for their insertion and, in addition, may carry genes for antibiotic resistance or for toxin production or may have no additional genes.

VI. **Genetic Exchange:** although recombination has been described, little has yet been said about how gene-bearing pieces of DNA are moved from one bacterial cell to another. There are three such processes of prokaryotic genetic exchange and are distinguished by the form of the donor DNA.

A. **Conjugation:** the donor cell contributes energy and building blocks to the synthesis of a new strand of DNA, which is physically transferred to the recipient cell. The recipient completes the structure of double stranded DNA by synthesizing the strand that complements the strand acquired from the donor. Plasmids are the genetic elements most frequently transferred.

Plasmids can also be regarded as accessory chromosomes. Plasmids are found frequently in dentally and medically important bacteria isolated from patients. These plasmids often carry genes for antibiotic resistance, and it is not unusual for a single isolate to be resistant to six or more antibiotics.

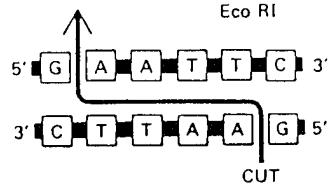
B. **Transduction:** donor DNA is carried in a phage coat and is transferred into the recipient by the mechanisms used for phage infection.

C. **Transformation:** the direct uptake of donor DNA by the recipient cell. The natural occurrence of this property is unusual among bacteria, and some of these strains are transformable only in the presence of **competence factors**, produced only at a specific point in the growth cycle.

VII. **Genetic Engineering:** The essential technologic advance which has allowed engineering based on bacterial genetics to transform biology derives from the ability of **restriction enzymes** to cleave DNA at sites determined by a specific oligonucleotide sequence to create **restriction fragments**. Simple techniques enable these fragments to be separated on the basis of size. The nucleotide specificity required for cleavage by restriction enzymes allows fragments containing genes or parts of genes to be covalently bound to plasmids that can then be inserted into bacterial hosts. Bacterial colonies or **clones** carrying specified genes can be identified by **hybridization** of DNA or RNA with various probes. Alternatively, protein products encoded by the genes can be recognized

either by enzyme activity or by immunologic techniques. It is therefore possible to use genetic engineering techniques to isolate virtually any gene with a biochemically recognizable property.

A. Cloning Of Restriction Fragments: restriction enzymes are used to create DNA fragments with cohesive (**sticky**) ends.



B. Vectors: restriction fragments can be placed in plasmids and introduced into a bacterial host. Drug resistance markers can be used to select the recombinant plasmids. Shuttle vectors have been designed to permit genes to be transferred among different microbial hosts.

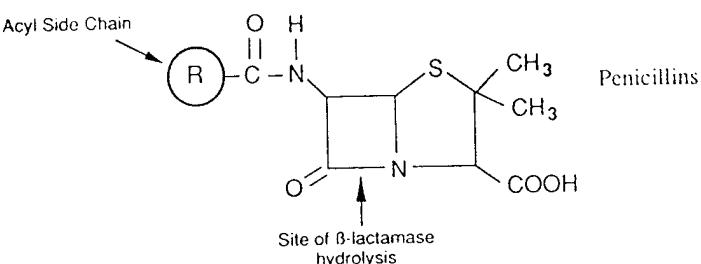
C. Manipulation Of Cloned DNA: Genetic engineering has been used to produce such useful substances as insulin, hum, an growth hormone, hepatitis B vaccine and others. It has also been used to study problems in oral biology. It is difficult or impossible to study genetics of the cariogenic bacterium *S.mutans* by transformation, transduction, or conjugation. Use of recombinant DNA technology has allowed the study of some *S.mutans* genes in *E.coli*, and there is beginning to be some understanding of the organization, function, and control of those *S.mutans* genes involved in caries formation. For example, these methods have been used to study inhibitors of glucosyltransferase in the absence of competing enzymes.

LECTURE 14: ANTIMICROBIAL DRUGS-MECHANISMS OF ACTION AND DRUG RESISTANCE

The most important concept underlying antimicrobial therapy is **selective toxicity**, ie, selective inhibition of the growth of the microorganism without damage to the host. Selective toxicity is achieved by exploiting the differences between the metabolism and structure of the microorganism and the corresponding features of human cells. There are 4 major sites in the bacterial cell that are sufficiently different from the human cell that they serve as the basis for action of clinically effective drugs: cell wall, ribosomes, nucleic acids, and cell membrane. If an ideal antibiotic were to exist it would be expected to be selectively toxic for microorganisms without injuring the host but in addition, it would be water soluble, active at physiologic pH, nonallergenic, not cause susceptible organisms to develop resistance, bactericidal, remain stable and active at bactericidal levels for long periods of time, and its activity would not be compromised by serum or pus.

I. Inhibition of Cell Wall Synthesis:

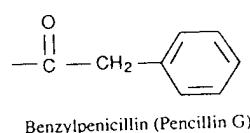
A. **Penicillin:** penicillin is not a single substance but a family of closely related entities that share a common nucleus and ring structure but differ in the nature of the side chain that is attached to the



nucleus. These antibiotics act by inhibiting **transpeptidases**, the enzymes that catalyze the final cross-linking step in the synthesis of peptidoglycan. There are 2 additional factors involved in the action of penicillin. The first is that penicillin binds to a variety of receptors in the bacterial cell membrane and cell wall called **penicillin-binding proteins (PBPs)**. Some PBPs are transpeptidases. The second factor is that **autolytic enzymes** called murein hydrolases are activated in penicillin-treated cells and degrade the peptidoglycan.

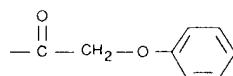
1. **Activity:** Narrow spectrum. Penicillin G (benzylpenicillin) is generally active against most gram-positive cocci, such as

staphylococci, streptococci, and pneumococci, but not against enterococci or β-lactamase-producing staphylococci. In addition, it is active against certain gram-negative cocci, such as gonococci and meningococci.



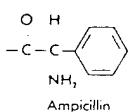
2. **Disadvantages:** First, cannot be taken orally because gastric acid inactivates it by hydrolyzing the β-lactam linkage. Second, the emergence of staphylococci that produced hydrolytic enzymes (penicillinases or β-lactamases) to attack the β-lactam linkage render penicillin G useless for treating such staphylococcal infections. Third, can cause the development of hypersensitivity. Finally, with the exception of certain oropharyngeal strains of *Bacteroides*, penicillin G has little or no activity against gram-negative rods.

3. Potassium Penicillin V: substitution of a phenoxyethyl group for the benzyl group. Because the addition of this group stabilized the β -lactam linkage in the presence of acid, this penicillin can be taken orally. Other disadvantages persist and the relative biologic potency of the antibiotic is decreased relative to penicillin G. Narrow spectrum.

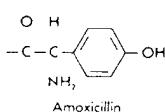


Phenoxyethylpenicillin (Penicillin V)

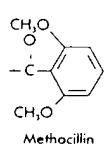
4. Ampicillin: the addition of an amino group to the benzyl ring of penicillin G, resulted in ampicillin. Ampicillin has the advantage that it can be given orally and it has an extended spectrum against the gram-negative organisms important in urinary and gastrointestinal tract infections. Also, its antibacterial activity approaches that of penicillin G. Broad spectrum.



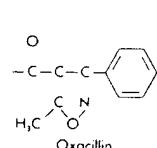
Ampicillin



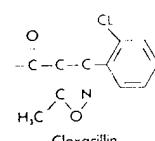
Amoxicillin



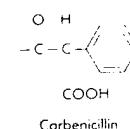
Methicillin



Oxacillin

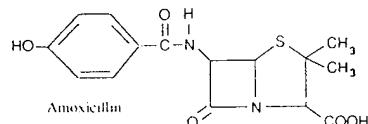


Cloxacillin

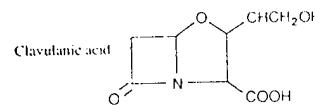


Corbenicillin

5. Augmentin: a mixture of amoxicillin and clavulanic acid. Amoxicillin is similar to ampicillin and offers an extended spectrum activity and acid stability. Clavulanic acid inactivates β -lactamases and permits it to be used in conditions where these enzymes are produced. Broad spectrum.

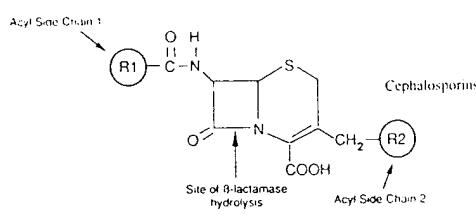


Amoxicillin



Clavulanic acid

B. Cephalosporins: these antibiotics work like penicillin but their structure is a little different. They are generally less effective than penicillin and do not cross the blood brain barrier and therefore not effective against meningitis. They are also poorly absorbed from the gut. Cephalosporins and penicillin have similar action on peptidoglycan synthesis but may differ in the selectivity of their binding to particular penicillin-binding proteins.



They are generally less effective than penicillin and do not cross the blood brain barrier and therefore not effective against meningitis. They are also poorly absorbed from the gut. Cephalosporins and penicillin have similar action on peptidoglycan synthesis but may differ in the selectivity of their binding to particular penicillin-binding proteins.

1. Cephadrine (Velosef): a semisynthetic extended spectrum antibiotic, especially against Gm negative bacteria. Less effective against β -lactamase positive organisms.

2. Cephalexin (Keflex): used for penicillinase producing staphylococci and in allergic patients. Active against actinomycetes

2. **Cephalexin (Keflex):** used for penicillinase producing staphylococci and in allergic patients. Active against actinomycetes and black-pigmented *Bacteroides*.

C. Mechanisms Of Drug Resistance:

1. **β -lactamases:** production of enzymes such as penicillinases, cephalosporinases, and amidases which destroy the antibiotic. Some are induced and secreted and others are constitutively produced and not secreted. They are mediated by plasmids (episomes).

2. **penicillin-binding proteins:** such proteins can be lost by mutation. An example is *Neisseria*.

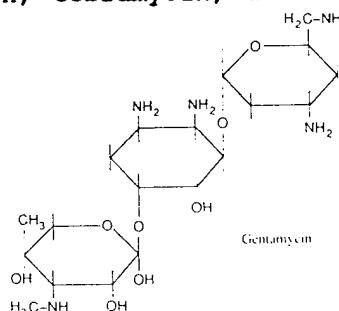
3. **tolerance:** bacteria can become tolerant to antibiotics, they are inhibited but not killed. Can result from a failure of activation of autolytic enzymes such murein hydrolase.

II. Inhibition Of Protein Synthesis-Action on 30S subunit

-Streptomycin, gentamicin, tetracycline

A. **Aminoglycosides:** The prototype of these antibiotics was streptomycin. Other aminoglycosides include neomycin, kanamycin, gentamicin, tobramycin, and amikacin. Two important modes of action of

aminoglycosides have been documented best for streptomycin; other aminoglycosides probably act similarly. Both inhibition of the "initiation complex" and misreading of messenger RNA occur. These antibiotics are bactericidal.

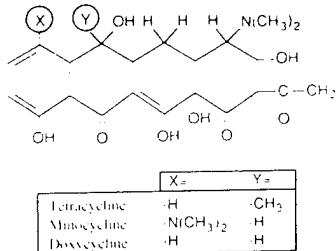


1. **Activity:** active against aerobic gram-negative bacilli, such as *E.coli*, *Proteus*, and *Klebsiella*. The aminoglycosides are among the most widely used antibiotics for serious gram negative infections.

2. **Disadvantages:** these antibiotics are ototoxic and nephrotoxic.

3. **Mechanisms of Drug Resistance:** Initiated by a. modification of the drugs by plasmid-encoded phosphorylating, adenylylating, and acetylating enzymes; b. chromosomal mutation; and c., decreased permeability of the cell to the drug

B. Tetracycline Antibiotics-doxycycline, minocycline: tetracycline and its analogs are considered to be broad-spectrum antibiotics. These antibiotics inhibit protein synthesis by binding to the 30S ribosomal subunit and blocking the aminoacyl transfer RNA from entering the acceptor site on the ribosome. However, selectivity is based on its greatly increased uptake into susceptible bacterial cells.



Structure of the tetracycline antibiotics

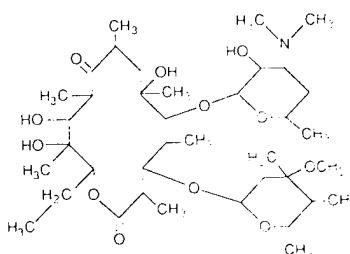
1. Activity: The spectrum of activity not only includes most gram-positive and gram-negative cocci and rods, but mycoplasma, rickettsiae, and chlamydia. They are usually the drug of first choice for disease caused by gram-negative organisms, such as cholera and brucellosis.

2. Disadvantages: adverse reactions include gastric disturbances predisposing to the overgrowth of other organisms in the oral cavity, vagina, and intestinal tract. The best example of this latter phenomenon is the appearance of oral or vaginal candidiasis following the administration of tetracycline. Tetracyclines also form chelates with calcium and related cations and can bind firmly to bone and teeth. Residues of tetracycline localize and persist in these tissues long after the drug is cleared from the body. As a result, severe permanent staining can occur in developing teeth. Finally, tetracycline, once one of the most widely used antibiotics, has the disadvantage that many resistant strains of microorganisms have emerged over the past few years.

3. Mechanisms of Drug Resistance: generally plasmid encoded effects on uptake or transport. If one antibiotic becomes inactive, all are inactive.

III. Inhibition Of Protein Synthesis-Drugs that act on the 50S subunit

A. Macrolide Antibiotics: Erythromycin-binds to the 50S subunit and blocks peptide bond formation by preventing the release of the uncharged transfer RNA from the donor site after the peptide bond is formed. One of the least toxic antibiotics.

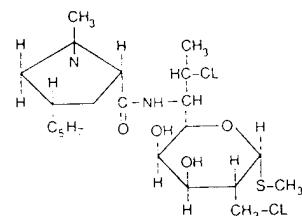


1. Activity: activity against gram-positive cocci, including β -lactamase-producing staphylococci. Erythromycin is considered to be a drug of initial choice in the treatment of infections caused by *Legionella*, *Bordetella pertussis*, *Mycoplasma pneumoniae*, and *Chlamydia trachomatis*. Ineffective against anaerobes producing dental infections such as most *Bacteroides*. Similar to penicillin V and a good alternative for common infections.

2. Disadvantages: disadvantages are few with the exception of gastrointestinal distress with oral use.

3. Mechanism of Drug Resistance: production of a plasmid-encoded enzyme that methylates the 23S ribosomal RNA and blocking binding of the drug.

B. Clindamycin: binds to the 50S subunit and blocks peptide bond formation



1. Activity: a spectrum that includes gram-positive and gram-negative cocci and some gram-positive rods, such as *Clostridium*, *Actinomyces*, and *Nocardia*. In addition, they are active against certain gram-negative bacilli, such as *Bacteroides fragilis* and *Bacteroides melaninogenicus*. Its most effective clinical use is against penicillin-resistant anaerobes. Bacteriostatic

2. Disadvantages: pseudomembranous colitis

3. Mechanisms of Drug Resistance: plasmid-encoded

IV. Inhibition Of Nucleic Acid Synthesis-Quinolones

A. Ciprofloxacin HCl-block bacterial DNA synthesis by inhibiting DNA gyrase

1. Activity: a broad spectrum antibiotic used for lower respiratory tract infections, urinary tract, bones and joints.

2. Disadvantages: the most common adverse reactions are gastrointestinal

3. Mechanism of Drug Resistance:

V. Alteration Of Cell Membrane Function

A. Nystatin-polyene antibiotic like amphotericin B disrupts the cell membrane of fungi due to affinity for ergosterol

1. **Activity:** fungistatic or fungicidal for a wide variety of yeasts

2. **Disadvantages:** insoluble and toxic when used parenterally. Not absorbed from the mucous membranes of GI tract.

3. **Mechanism of Drug Resistance:**

B. **Clotrimazole-** imidazole antibiotic like ketoconazole

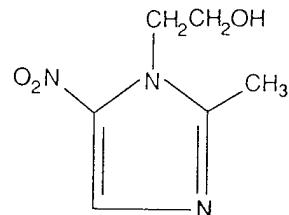
1. **Activity:** Broad spectrum anti-fungal

2. **Disadvantages:** Toxic to the liver

3. **Mechanism of Drug Resistance:**

VI. Uncertain Mechanisms Of Action

A. **Metronidazole-**acts as an electron sink and deprives organisms of needed reducing power. It also binds to DNA and causes strand breakage



1. **Activity:** Antiprotozoal and useful for obligate anaerobic bacteria responsible for Vincents infections

2. **Disadvantages:** nausea, vomiting and teratogenic?

3. **Mechanisms of Drug Resistance**

VII. Nongenetic Basis Of Resistance

1. bacteria can be walled off within an abscess cavity which the drug cannot penetrate
2. bacteria can be in a resting state and insensitive to cell wall inhibitors. Mycobacterium can remain dormant in tissues
3. organisms can survive as protoplasts
4. several artifacts that can make it appear that the organisms are resistant, eg, administration of the wrong drug or the wrong dose, failure of the drug to reach the appropriate site in the body, improper administration of the drug, or failure of the patient to take the drug.

Typical Examination Questions:

1. Discuss the basic differences between gram positive and gram negative cell walls and comment critically on the following statement: Bacteria are either gram positive or gram negative.
2. Identify five (5) bacterial structures and indicate their location in the bacterial cell as well as their function(s). In particular, indicate which structures are important in bacterial induced pathogenesis.
3. Briefly discuss the differences between aerobic and anaerobic respiration and fermentation.
4. List the four major ecosystems into which the oral cavity can be divided and suggest three reasons as to why the indigenous flora might be unique in these areas.
5. Briefly discuss the nature of the ecological succession of the oral microflora during the first 12 months of life.
6. Identify ecological determinants including environmental factors that influence microbial acquisition.
7. Discuss how the streptococci are differentiated and list the main groups. Which groups are most important as oral microflora and why?
8. Differentiate among those viruses which present major risks to the dentist
9. Identify five substances or structures that increase bacterial invasiveness and briefly discuss its major characteristics
10. Discuss the possible consequences in a bacterial infection of the transmission of plasmids
11. Differentiate between the properties of endotoxin and exotoxin and give examples of bacteria producing each
12. Discuss the nature of the salivary pellicle and its role in the initiation of plaque development
13. Discuss the role of host factors in defining the bacterial composition of plaque.
14. Discuss the nature of the unique environment set up by plaque and how this environment influences the microorganisms taking part.

15. Discuss the basic principles involved in coaggregation and indicate how specific bacterial interactions can come about even though the bacteria may fail to recognize each other directly.

16. Critically comment on the following statement: It would be expected that oral bacteria resident in plaque would have similar growth characteristics as those growing on defined bacterial media.

17. Briefly discuss the concept of sucrose dependent virulence relative to caries formation.

18. Briefly identify and characterize those viruses which present major risks to the dentist.

19. Discuss the genetic versus infectious controversy of the etiology of caries and how it was settled.

20. Discuss the various characteristics of *S. mutans* and in particular those which provide the potential to form caries.

Typical Examination Questions cont.
Dental Microbiology 1992

21. Respond critically to the following statement: "It is well known that *Streptococcus mutans* is the etiologic agent of dental caries!"
22. Briefly discuss the major conceptual advances which resulted in the identification of *Streptococcus mutans* as the etiologic agent of coronal caries.
23. Discuss the concept of dental focal infection. Define the concept and give reasons as to why the concept is important. In addition, suggest four types of systemic infections where the phenomena is thought to occur.
24. List and briefly discuss the various characteristics of *Porphyromonas gingivalis* which would give it the necessary ability to participate in the development of periodontal diseases. Be sure to include elements of colonization, evadence, and tissue destructive ability.
25. Summarize the evidence for a bacterial etiology of periodontitis.
26. Briefly summarize the major similarities and differences regarding the types of flora found in health, gingivitis, and periodontitis.
27. Critically discuss the following statement: "It is well accepted that the specific plaque hypothesis of periodontitis has been proved; after all adult periodontitis is caused by *P. gingivitis*."
28. As a result of a variety of circumstances dental pulp may become exposed to bacteria. Discuss how this can come about and indicate the results of such exposure. Your answer should include a sampling of the organisms involved.
29. Indicate reasons as to why the establishment of periodontal disease is not guaranteed if a periopathogen is inoculated into gingival tissue.
30. Summarize the microbial virulence determinants thought to be involved in periodontal disease.
31. In a general way discuss the ecological attributes of endodontal infections. Are their similarities with ecological succession seen in dental plaque development.
32. Discuss how the process of anachoresis can result in systemic infection.

33. Discuss how endocarditis can come about as a result of an oral infection.

34. Genetic engineering technology has been used extensively in biomedical research over the last ten years. This technology is currently being applied in various aspects of dental research and in particular to understand the role of microbes in the establishment of dental caries and periodontal diseases. What is the value of placing DNA from oral bacteria or periopathogens in *E. coli* and what are some examples of information that may be obtained.

35. Discuss the general nature of plaque development including the predominant microorganisms found at various stages. Your discussion should also address aspects of the chemical composition of plaque, unique aspects of bacterial attachment, and the influence that the unique oral environment has on the physiology of organisms taking part. Be sure to also identify the various host factors involved.

36. Discuss the concept of selective toxicity and give five examples where this has been applied in the development of antibiotics. Your response should include a discussion of the similarities and differences between microorganisms and indicate why some antibiotics are more toxic to human tissues than are others.

37. Comment critically on the following statement: "It makes perfect sense to find polymicrobial infections to be common in root canals".

38. What is meant by the concept of a "multifactorial infectious disease". Give two examples of such diseases occurring in the oral cavity and identify the factors involved.

39. Identify salivary factors that might have a positive influence on the growth and development of oral microbes.

40. How might it be possible to have a situation in which three individuals are known to be infected with *Actinobacillus actinomycetemcomitans* and yet only one convert to a disease state (development of JP).



EVIDENCE FOR SUPERANTIGEN PRODUCTION BY BACTERIA ASSOCIATED WITH ADULT PERIODONTITIS

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ABSTRACT

Anaerobic bacteria such as *Porphyromonas gingivalis* (*P. g.*), *Prevotella intermedia* (*P. i.*) and *Actinobacillus actinomycetemcomitans* (*A. a.*) produce immunomodulatory factors including polyclonal B-cell activators. In this study we evaluate superantigen properties of lysates from *P. g.*, *A. a.* and *P. i.*. Bacteria were disrupted in a bead mill and extracts prepared. Peripheral blood mononuclear cells (PBMC) or purified T-cells cultured in the presence or absence of antibody to MHC determinants were incubated with bacterial extracts. Cells were harvested and proliferative determination by thymidine incorporation. Anti-HLA Class II antibody inhibited cell proliferation. IL-2 production was stimulated by bacterial antigens. Results from flow cytometry studies utilizing specific antibody to various T-cell receptor Vβ determinants as well as quantitative rt-PCR studies have also been utilized to evaluate superantigen production by *P. g.* and *A. a.* Co-culture studies utilizing purified B-cells and T-cells previously exposed to toxic shock syndrome toxin suggests that a superantigen dependent polyclonal B-cell activation process also exists. Supported by NMRDC Work Unit 0601152N/MR00001.001-0063.

INTRODUCTION

Numerous studies support the contribution of B-cells to the pathogenesis of periodontal diseases. A great deal of confusion still exists, however, regarding the role of T-cells. Work presented here suggests that superantigens (SA) are present in extracts from anaerobic periodontal pathogens.

METHODS

PBMC were co-cultured with extracts obtained from periodontal pathogens. To estimate TCR-Vβ gene expression, PBMC mRNA was reverse transcribed and the cDNA was amplified using 5'Vβ-specific sense primers and a 3' Cβ-specific antisense primer. As an internal control, TCR Cα cDNA was co-amplified in each reaction mixture using a 5'Cα sense primer and 3' antisense primer. Products were evaluated with an ABI sequencer.

TCR-Vβ expression on CD3+ lymphocytes was measured by 2- and 3-color flow cytometry. Vβ expression was measured with FITC-labeled monoclonal antibodies (Immunotech Inc.).

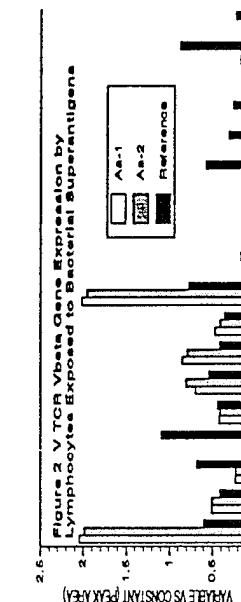
Toxic shock syndrome toxin-1 (TSST-1) reactive CD4+ cells were isolated using a Percoll separation technique. This population was used rather than an unselected resting T-cell population to optimize conditions for detecting B-cell activation. B-cell enriched populations were prepared by rosette formation followed by complement-mediated lysis of contaminating T-cells. Responding B-cells were cultured in medium alone or with fresh autologous T-cells or CD4+ TSST-1 reactive cells. Where indicated, cultures were supplemented with Staphylococcal Protein-A or TSST-1. T-cells were treated with mitomycin C. Polyclonal responses were determined by evaluating hemolytic plaque formation to FITC-labeled SRBC.

RESULTS

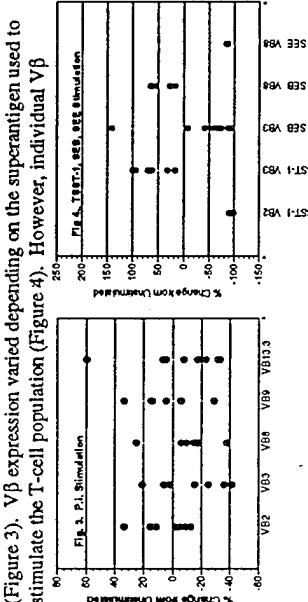
Typical gels for evaluating amounts of Vβ mRNA production are shown in Figure 1. Figure 2 shows increased expression of TCR-Vβ I and TCR-Vβ8 after stimulation by *A. a.*

Evaluation of the influence of antibody to Class I and Class II MHC components indicate that MHC Class II(DR) determinants inhibit lymphocyte proliferation (Figure 5). Antibody to MHC Class I(B) did not inhibit proliferation.

We also evaluated the possibility of a superantigen-dependent polyclonal B-cell (PBA) activation response mediated by CD4+ TSST-1 reactive T-helper cells. In Figure 6, peripheral blood B-cells were cultured either alone, with autologous CD4+ TSST-1 reactive T-cells, or with autologous T-cells, or with analogous CD4+ TSST-1 reactive T-cells. Cultures were supplemented with Staphylococcal Protein A or TSST-1.



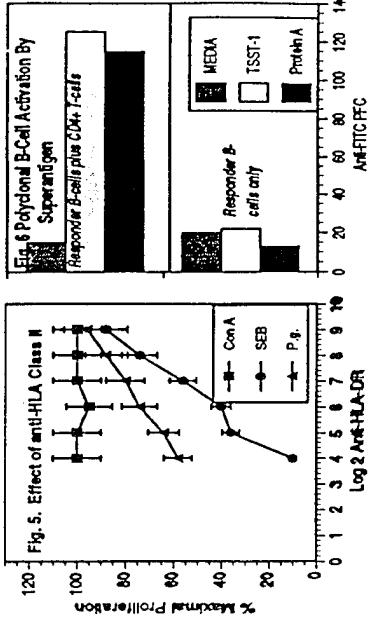
Changes in Vβ expression following stimulation with either crude homogenates of *P. i.* or purified superantigen were analyzed using flow cytometry. The expression of Vβ 2, 3, 8, 9, and 13.3 after incubation with crude homogenates of *P. i.* was highly variable among the 9 subjects tested (Figure 3). Vβ expression varied depending on the superantigen used to stimulate the T-cell population (Figure 4). However, individual Vβ



CONCLUSIONS

Utilizing rt-PCR methodology we have shown that *A. a.* extracts differentially stimulate TCR-Vβ1 and Vβ8 mRNA amounts. In addition, we have demonstrated that a portion of the lymphocyte response to such components are dependent on interaction with cells bearing Class II MHC antigens. These observations suggest that SA or SA-like activities are present in periodontal pathogens.

Of particular significance is our finding that SA may induce polyclonal B-cell responses in the absence of PBAs. It is possible, therefore, that a bridging mechanism exists involving B-cells and SA which results in enhanced differentiation of B-cells. This, in addition to B-cell stimulation initiated by known bacterial PBAs, could be responsible for the abundance of B-cells in advanced periodontal lesions.

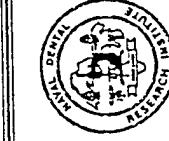


SUPERANTIGEN AND IL-2 RELATIONSHIPS IN PERIODONTITIS

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Cytokines and superantigens appear to participate in the pathogenesis of periodontitis. However, little is known regarding differences in susceptibility to periodontal tissue breakdown and variations in cellular cytokine responses to etiologically important bacteria. This study examines the relationship of IL-2 production by peripheral blood mononuclear cells (PBMC) from diseased and non-diseased patients after culture with *Treponema denticola* (T.d.) antigen or specific T-cell stimulators. PBMC were cultured with or without T.d. or the superantigens SEA or TSST-1. Cell proliferation was assessed by evaluation of ^3H -TdR incorporation. IL-2 released into culture supernatants was determined by ELISA. IL-2 production and cell proliferation were significantly elevated in PBMC from diseased subjects which had been cultured with T.d. (ATCC 33521). IL-2 was elevated in non-diseased subjects which were cultured with SEA. TSST-1 did not result in significant differences in IL-2 production when diseased and non-diseased subjects were compared. These results support the concept that individuals with severe periodontal disease may have been exposed to superantigens specific for some or all of the TCR-V beta determinants recognized by SEA but may not have been exposed to superantigens specific for TCR-V beta determinants recognized by TSST-1. Therefore, it is possible that T.d. (ATCC 33521) carries non-TCR-V beta 2 specific superantigens. The presence of superantigens in T.d. are currently being evaluated using quantitative rt-PCR procedures. Supported by NMRDC Work Unit 0601152N.MR00001.001-0063.



FUNCTIONAL AND AUTOLOGOUS IMMUNE RESPONSES OF CRYOPRESERVED HUMAN LYMPHOCYTES

T. Canaan^{*1}, M. Hickey¹, A.W. York¹, and G.A. Miller^{1,2}(Naval Dental School¹, Bethesda, MD and Geo-Centers, Inc.², Fort Washington, MD)**ABSTRACT**

Various studies have indicated that freezing techniques provide a means of repeating and extending immunological assays using frozen stored portions of the same lymphocyte cell sample. We and others have demonstrated that short-term frozen and stored lymphocytes retain their ability to respond to antigens of dental interest as well as to polyclonal B-cell activators (PBA) and superantigens (SA). The objective of this study was to extend our previous findings by determining whether frozen and stored lymphocytes retained functional activity after more than 50 weeks in frozen storage. In addition, studies were conducted to determine whether the autologous immune response demonstrated by unstimulated lymphocytes also retained activity. Accordingly, peripheral blood mononuclear cells (PBMC) from four normal human subjects were frozen using a Cryomed Model 1010 programmable freezing system and stored in liquid nitrogen. At intervals thereafter, portions of each sample were assayed for biogenic responses and cytokine production after stimulation by a variety of lymphocyte stimulators including PBA, SA, and bacterial extracts. Our results indicate that frozen and stored lymphocytes retained viability of functional activities even after more than a year in liquid nitrogen storage. The most constant responses were observed among autologous immune responses. Frozen storage continues to represent a viable method to permit calendar immunobiological assays on clinically characterized subjects. Supported by NMRDC Work Unit 0601152N.NR00001.001-0063.

BACKGROUND

Several studies suggest an important role for PBA in the pathogenesis of periodontal disease. SA may also play a role in the development of periodontal inflammatory reactions. Since periodontitis is a dynamic process, constant alterations occur in the immune status of characterized patients with the advancement and regression of disease. Longitudinal evaluations of immunological responsiveness in periodically characterized patients, including comparisons of their responses with those of control patients with a healthy periodontium, would help us to better understand the immune modulators involved in this insidious disease process. The difficulty with such studies is the lack of appropriate controls and variability inherent in many assay systems. A method which allows for the storage of cells and comparison of future samples against a standard control is cryopreservation. Cellular assays can be extended and repeated on characterized subjects, evaluating the functional activity of lymphocytes in response to various stimulators. Our previous studies focused on the proliferative responses of lymphocytes showing that they do retain their ability to respond to various antigens following frozen storage. The functional activity of lymphocytes must also be evaluated, however. Since IL-1 β is an important mediator in periodontal inflammation and because it also provides information concerning the functional activity of lymphocytes following frozen storage, we chose to evaluate this particular cytokine.

METHODS

PBMC were obtained from fresh heparinized whole blood from

four healthy adults. The whole blood was mixed 2:1 with Hank's Balanced Salt Solution (HBSS); peripheral blood lymphocytes (PBL) were separated using Ficoll-Hypaque density centrifugation and washed in RPMI-1640 medium containing 10% heat activated pooled human A or AB plasma. Viable cells were transferred to standard culture medium; cells to be frozen were diluted and transferred to chilled freezing medium and frozen using a Cryomed programmable cell freezer. Frozen cells were stored in liquid nitrogen.

The thawing procedure was accomplished by rapid transfer of frozen cell samples from storage to a 37°C water bath followed by sustained agitation. Thawed samples were placed in an ice bath; the contents of each vial were transferred to 1.5 ml tubes and diluted in defrosting medium.

After centrifugation, viability was assessed by phase-contrast microscopy and trypan-blue exclusion. Each vial was treated as if it contained 10 \times 10 6 cells and then diluted appropriately for assay. One hundred microliter aliquots were plated into wells containing 20 μ l of appropriate activator. Triplicate wells were prepared for background and each activator concentration. Cultures were then covered and incubated at 37°C in a 5% CO₂ atmosphere for 4 days and the level of proliferation determined by incorporation of ^{3}H dThR. Supernatants were collected prior to assay for blastogenic responses. Commercial enzyme-linked immunosorbent assay kits were used to determine IL-1 β production.

Bacterial stimulants were prepared in the following manner. The strains of bacteria used were *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. After culture, cells were washed in PBS, weighed, and placed in 1 part glycerol and 2 parts PBS mixture to give 0.1 gm of cells/ml of suspension and then frozen. Bacteria were thawed, washed three times in PBS, and subjected to bead mill homogenization. Disrupted whole cell suspensions were used to stimulate PBMC cultures in the assays. Superantigens, mitogens, and a polyclonal D-cell activator were prepared from lyophilized preparations by suspension and dilution in RPMI-1640 to the desired concentrations.

RESULTS

Freezing had no effect on the activities measured. Figures 1, 2, and 4 show cell proliferation responses of subjects to various stimulators at different time intervals. Spontaneous proliferation was evaluated during the course of most blastogenic transformation studies and is shown in Figure 3. Peak response time was found to be 7-9 days. The functional activity of cells at week 0, 12, and 22 is demonstrated in Figure 5 by IL-1 β production in response to various stimulators.

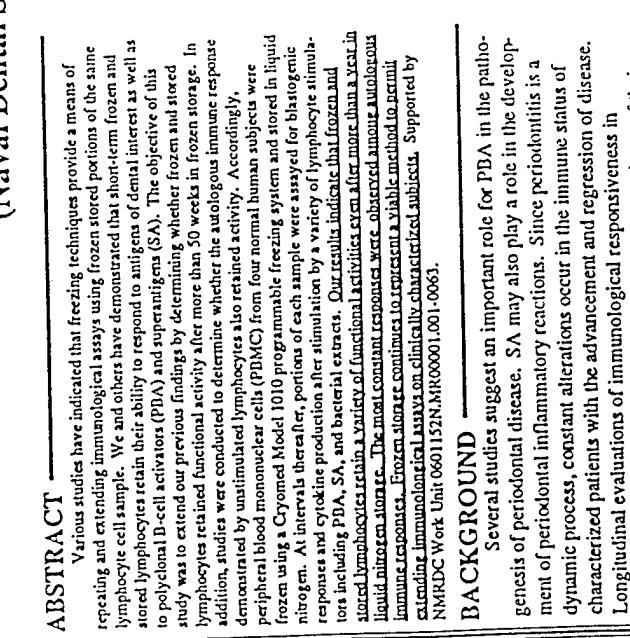
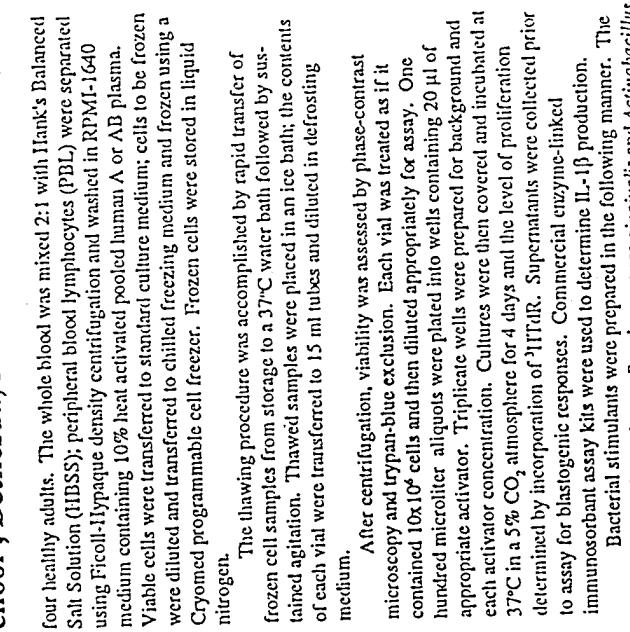
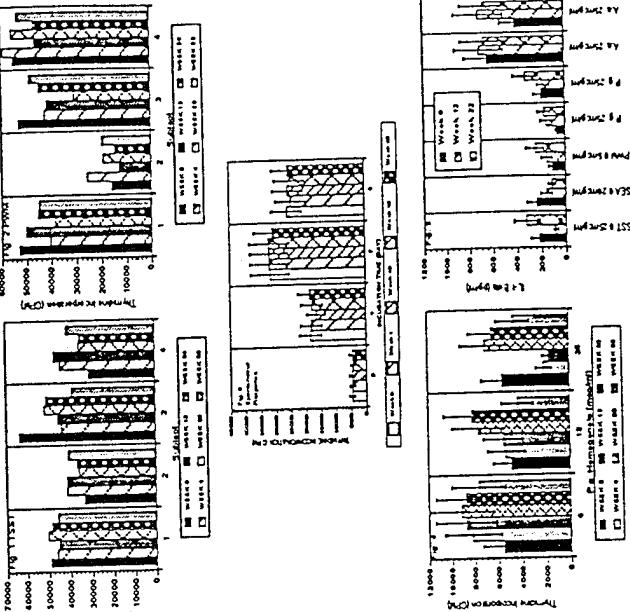
DISCUSSION

This study shows that lymphocytes can be frozen and stored for extended periods of time and retain their blastogenic responses as well as a variety of functional activities. Frozen storage continues to be a convenient means of storing cells and maintaining viability and immunologic responsiveness. This tool may prove useful in our future understanding of the role immune modulators play in periodontal diseases, and may help to eliminate some of the variability inherent in some cellular assays.

CONCLUSIONS

1. The blastogenic response of PBMC following frozen storage remained constant.
2. Freezing had no effect on PBMC ability to undergo spontaneous proliferation.
3. The functional activity of PBMC, as measured by the production of IL-1 β , showed no effect from frozen storage.

Supported by NMRDC Work Unit 0601152N.NR00001.001-0063.





EVIDENCE FOR SUPERANTIGEN PRODUCTION BY BACTERIA ASSOCIATED WITH ADULT PERIODONTITIS

G. A. Miller^{1,2} and D.C. Alexander¹ (Naval Dental School¹, Bethesda, MD and Geo-Centers, Inc.², Fort Washington, MD)

ABSTRACT

An *autoimmune* factor such as *Porphyromonas gingivalis* (*Pg*) have been shown to produce immunomodulatory factors such as mitogenic and polyclonal B-cell activators. The purpose of this study was to evaluate superantigen properties of lysates from *Pg*. Pooled bacterial cultures were disrupted in a bead mill and the whole cell homogenates were retained. Peripheral blood mononuclear cells (PBMC) or purified T-cells cultured in the presence or absence of anti-IL-2R antibodies. IL-2 was determined using a commercially available ELISA procedure.

RESULTS

In a large number of studies we observed that *Pg* and *Actinobacillus actinomycetemcomitans* (*aa*) bacterial homogenates were able to stimulate significant blastogenic responses in PBMC cultures (Figure 1). A portion of these responses was found to be due to polyclonal B-cell activation. In addition, *Pg* selectively increases the number of cells expressing the Vβ1, 13, 11, 17, and 19 mabs by two to five fold. These results suggest that *Pg* and *aa* produce molecules with some selective immunological properties.

METHODS

Bacterial homogenates were prepared with the aid of a bead mill from pooled cultures grown under anaerobic conditions in Wilkins-Chalgren broth supplemented with hemin and menadione. Whole cell homogenates were collected and in some instances, semi-purified protein constituents were obtained from bacterial homogenates by electroelution constituents from PAGE.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood using a Ficoll-Hypaque density gradient. Extract induced cell proliferation was monitored by radio-thymidine incorporation in the presence or absence of complement and antibody to CD4. Purified CD4+ T-cell populations were prepared using enrichment columns. To optimize conditions for detecting B-cell activation, toxic shock syndrome toxin-1 (TSST) reactive CD4+ cells were analyzed rather than unselected resting T-cell populations. PBIL were cultured with TSST. After 3 days, cultures were supplemented with IL-2 and 24 hours later, blast and resting cell separated by centrifugation on discontinuous Percoll gradients. TCL were maintained in IL-2 and expanded by weekly retriggering with TSST plus mitomycin-C treated autologous PBMC.

B-cell enriched populations were isolated from fresh PDL. T-cells were removed by rosette formation with neuraminidase-treated sheep erythrocytes, followed by complement-mediated lysis of contaminating T-cells by a monoclonal antibody to CD3. Resting D cells were isolated from monocytes and activated cells by density centrifugation on discontinuous Percoll gradients. Responder B-cells were cultured in round-bottomed culture tubes in 0.5 ml of final medium alone or with fresh autologous T-, autologous CD4+ TSST-reactive cells. Where indicated, cultures were supplemented with Staphylococcal Protein A or TSST. TLC populations were treated with mitomycin-C to prevent overgrowth, a procedure that does not affect helper activity. After 10

days, cultures were terminated and supernatants assayed for total IgM by ELISA or for hemolytic plaque formation to FITC-labeled SRBC.

The influence of antibody to MHC determinants was determined by culturing semi-purified bacterial components with PBMC in the presence or absence of anti-IL-2R antibodies. IL-2 was determined using a commercially available ELISA procedure.

RESULTS

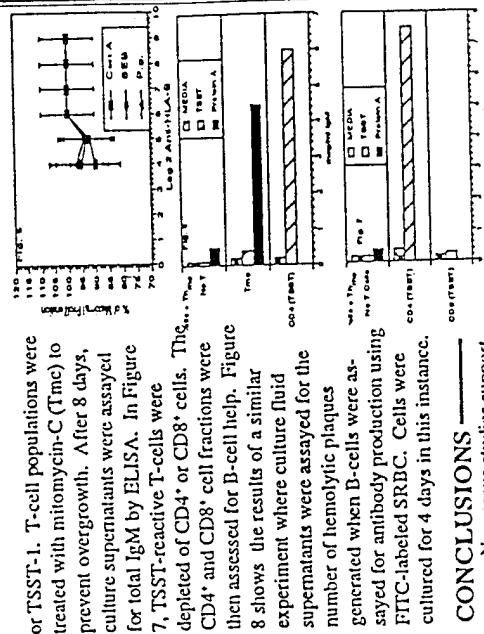
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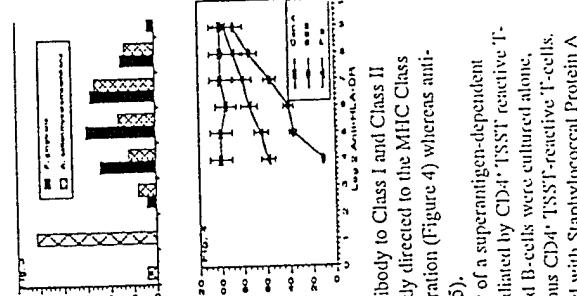
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or TSST-1. T-cell populations were treated with mitomycin-C (1mc) to prevent overgrowth. After 8 days, culture supernatants were assayed for total IgM by ELISA. In Figure 7, TSST-reactive T-cells were depicted of CD4+ or CD8+ cells. The CD4+ and CD8+ cell fractions were then assessed for B-cell help. Figure 8 shows the results of a similar experiment where culture fluid supernatants were assayed for the number of hemolytic plaques generated when B-cells were assayed for antibody production using FITC-labeled SRBC. Cells were cultured for 4 days in this instance.

CONCLUSIONS

Numerous studies support the contribution of B-cells to the pathogenesis of periodontal diseases. A great deal of confusion still exists, however, regarding the role of T-cells. Our recent studies suggest the possibility that superantigens are present in extracts from periodontopathogens. This is indicated by the presence of added polyclonal B-cell activators (Figures 6-8). It is possible, therefore, that a bridging mechanism exists involving B-cells and superantigens and can result in enhanced differentiation of B-cells. This, in addition to B-cell stimulation initiated by known bacterial PBAs, could be responsible for the large concentration of B-cells present in advanced periodontal lesions.



addition, by pretreating cells with antibody to CD3 and complement, a significant portion of the blastogenic response could be eliminated (Figure 2). In addition, purified T-cells failed to respond to the semi-purified bacterial extracts (Figure 1). We have recently found that such bacterial components were also able to induce significant IL-2 production by PBMC (Figure 3). From these results and others, we reasoned that this was the consequence of T-cell stimulation and therefore could possibly result from the presence of superantigens in the bacterial preparations. In preliminary studies, we evaluated the influence of antibody to Class I and Class II MHC components and found that antibody directed to the MHC Class II (DR) determinants could inhibit proliferation (Figure 4) whereas antibody to MHC Class I (D) do not (Figure 5).

We also evaluated the possibility of a superantigen-dependent polyclonal B-cell activation response mediated by CD4+ TSST-reactive T-cells. In Figure 6, peripheral blood B-cells were cultured alone,

with autologous T-cells, or with autologous CD4+ TSST-reactive T-cells.

As indicated, cultures were supplemented with Staphylococcal Protein A

Trypsin-like activity levels of *Treponema denticola* and *Porphyromonas gingivalis* in adults with periodontitis

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D. E. Chadwick³, P. J. Covill¹,
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Pederson ED, Miller JW, Matheson S, Simonson LG, Chadwick DE, Covill PJ, Turner DW, Lamberts BL and Morton HE: Trypsin-like activity levels of *Treponema denticola* and *Porphyromonas gingivalis* in adults with periodontitis. *J Clin Periodontol* 1994; 21: 519–525. © Munksgaard, 1994.

Abstract. *Treponema denticola* (Td) and *Porphyromonas gingivalis* (Pg) are associated with human moderate and severe adult periodontal diseases. This study quantifies these two anaerobes and their trypsin-like (TL) activities in subgingival plaque collected from both clinically healthy and periodontally diseased sites of human periodontitis patients. Antigen levels of the microorganisms were determined by monoclonal antibodies and TL activities were measured by the fluorescent substrate Z-gly-gly-arg-AFC in a disc format. Significant positive correlations were observed between the antigen levels and the TL activities when the data were subjected to statistical analyses both on a site-specific and on a patient basis. Anaerobe synergism was found between Td and Pg in a continental US population, and positive correlations were found between anaerobe levels (individually and total) and clinical indicators of adult periodontitis.

Positive correlations have been established among pocket probing depth (PD), connective tissue attachment loss, and indices of periodontal diseases in patients with a prevalence of subgingival treponemes (Listgarten & Levin 1981). *Treponema denticola* (Td) and four other oral treponemes were found to be among the most likely causative agents of moderate adult periodontitis (Moore et al. 1983), and Td antigen levels were elevated in subgingival plaque (SP) samples obtained from adults with severe periodontitis (Simonson et al. 1988a). Furthermore, Simonson et al. (1992a) provided evidence that the repopulation of periodontal pockets with Td can serve as a prognostic indicator of periodontal disease recurrence.

Bacteroides species have been recovered in high proportions from patients with advanced periodontitis (Slots 1979, Tanner et al. 1979). *Porphyromonas gingivalis* (Pg) (Shah & Collins 1988), formerly *Bacteroides gingivalis*, has been

shown to be more prevalent in periodontal sites sampled from diseased subjects than from non-diseased subjects (Dahlen et al. 1992). Pg has also been found to be associated with the adult form of periodontitis by Loesche et al. (1985) and Bretz et al. (1990).

Several of the gram-negative oral microorganisms associated with periodontal diseases elaborate trypsin-like (TL) enzymes (Loesche 1986, Loesche et al. 1990a). This characteristic may enhance their virulence (Slots 1981, Laughon et al. 1982, Pederson et al. 1988, Bretz et al. 1990) and could possibly serve as a marker to identify patients at risk of active adult periodontitis. Radford et al. (1992) have found TL activities in gingival crevicular fluid to be higher in subjects with adult chronic periodontitis than in subjects with gingivitis. These activities were reduced significantly by periodontal treatment.

Some clinical studies of TL enzymes (Loesche et al. 1990a, Watson et al. 1991,

Key words: trypsin-like activity; *Treponema denticola*; *Porphyromonas gingivalis*; periodontitis.

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Loesche et al. 1992) have demonstrated correlations between TL activity and the presence of specific microorganisms, such as Pg and Td. However, these studies involved the use of polyclonal antibodies, with oral sites being evaluated simply as positive or negative with respect to the presence of Pg or Td.

The present report has extended and lent support to the findings of these foregoing studies by the use of monoclonal antibodies, specific for Pg and Td, to permit the quantitation of these microorganisms in SP samples. Data from such measurements has provided new information on synergistic relationships of Pg and Td, and revealed significant correlations among levels and TL activities of these microorganisms in SP and clinical criteria of adult periodontal diseases.

Material and Methods

Patient selection and clinical status

Patients, 20 to 55 years of age, were selected from those referred to the De-

partment of Periodontics, Northwestern University Dental School, and were categorized into two groups (A and B).

Group A of 36 subjects included 22 males and 14 females, who were determined to be of case types I ($N=8$), II ($N=12$), or III ($N=16$) by the American Dental Association (ADA) classification criteria (Grant et al. 1988). Group B included 10 subjects (5 male and 5 female) who had PDs of 3 to 9 mm, were of ADA case types III ($N=6$) or IV ($N=4$) and met the above selection criteria. All subjects were free of medical complications and had not taken medication which could affect their periodontal status within the six months preceding the sampling. The gingival index (GI) of Löe & Silness (1963) was employed to assess gingival inflammation, and the Michigan periodontal probe was used to determine PDs.

Sample collection

Group A subjects provided 283 SP samples, each of which was used for determinations both of TL activity and of microbial counts. The SP was removed by a Gracey 7/8 curette from the mesial or distal surfaces of the maxillary right and left 1st and 2nd molars, and the maxillary right and left second premolars. SP was also removed by a single curettage from two additional sites which appeared to be stable and healthy. Each SP sample was placed in a 1.5 ml polypropylene microcentrifuge tube containing three or four 3-mm diameter glass beads and 100 μ l of collection buffer, composed of 150 mM NaCl + 20 mM MgCl₂, pH 7.5. All samples were collected within a 5-week period and stored frozen at -75°C prior to analysis.

Group B subjects provided 93 additional SP samples which were used solely for TL activity determinations. The SP was removed by a single curettage with a Gracey 7/8 curette. Each SP sample was placed in a 0.25 ml polypropylene microcentrifuge tube, without collection buffer, and was immediately frozen. All samples were stored at -75°C prior to analysis.

Substrate-impregnated disc preparation

The synthetic peptide substrate Z-gly-gly-arg-7-amino-4-trifluoromethyl coumarin (AFC) of Smith (1983) (Enzyme Systems Products, Livermore, Califor-

nia, USA), which is specific for trypsin, was solubilized, used to saturate sheets of filter paper (Whatman, Hillsboro, OR, USA) and the filter paper was air-dried. Quarter-inch AFC discs were prepared from the impregnated filter paper with a paper punch. These were then stored dry and protected from light at ambient temperature.

Culture of anaerobes

Treponema denticola isolates ATCC 33520, ATCC 33521, ATCC 35405, Ichelson, D65BR1, TD2, T32A and D39DPI were grown in 1186 broth (Jacob et al. 1980) under anaerobic conditions at 37°C for 3-5 days. *Porphyromonas gingivalis* isolates ATCC 33277, 238, 376, D40C4, D67D9, D43A5, D82F5, and JKG 5 were grown in Wilkins-Chalgren broth, containing 5 mg/l hemin and 0.05 mg/l menadione, for 3-5 days under anaerobic conditions at 37°C. The cells were harvested by centrifugation of 500 ml cultures at 10,400 $\times g$ for 15-20 min. The cells were washed once with 2 ml of the collection buffer and resuspended in 2 ml of this buffer.

Trypsin-like activity determination

The TL activity was determined essentially as reported by Cox et al. (1990). Each SP sample from group A subjects was vigorously mixed and 50 μ l of the suspension was placed directly onto an AFC disc. Each SP sample from group B subjects was mixed with 12 μ l of the collection buffer in its storage tube and compacted by gentle centrifugation (1000 $\times g$). The resulting pellet, the supernatant, and a 15 μ l rinse of each tube were combined on a single AFC disc for TL activity determination. By this procedure, the SP samples from group B subjects more closely represented the total SP than the SP samples from group A subjects. As noted above, with these latter subjects only half of the original SP sample was analyzed for TL activity.

The fluorescence was allowed to develop at ambient temperatures (25.0-27.5°C) for 5, 10, 15, 30, and 60 min following sample application. The discs, evaluated under ultraviolet light at 366 nm, showed colors ranging from a deep blue for no TL activity (blank), or a light blue for slight TL activity, to bright yellow-green for high activity. No inhibitors to trypsin were included in

either the collection buffer or the filter paper discs as in the earlier Cox and Eley studies (1989a & 1989b).

Fluorescence standards were prepared by incubations of 5 to 50 pg of bovine pancreatic trypsin III (Sigma Chemical Co., St. Louis, MO) on the AFC discs for 5, 15 and 30 min, and the reactions were stopped with buffered formalin. The standards ranged from 0 for no fluorescence to 3 for the bright yellow-green color, with 0.5 unit increments. Two observers visually scored the fluorescence of the test samples against these standards.

Serial 1:2 dilutions of the cultured organisms were prepared and 10, 25, 50 and 100 μ l of each dilution were applied to the AFC discs for TL-activity evaluation. The number of cells needed to yield a visually detectable fluorescence after 5 min of incubation was determined. This number probably included both viable and non-viable cells since the TL activity persists following cell death.

Treponema denticola and *Porphyromonas gingivalis* ELISA

The presence of Td and Pg in the remainder of the SP samples from group A patients was determined by an enzyme-linked immunosorbent assay (ELISA) developed by Simonson et al. (1988a), using monoclonal antibodies specific for these oral anaerobes (Simonson et al. 1986, 1988b & 1990). Two hundred μ l of the NaCl + MgCl₂ buffer containing 1% formalin was added to the 50 μ l of each SP sample remaining following TL activity determinations. This suspension was vigorously mixed in the presence of glass beads and 100 μ l portions were used for determining mg total SP. The wet weight of the SP was calculated by measuring absorbance of the SP suspension at 420 nm. Absorbance values were compared with a standard curve prepared from serial dilutions of preweighed pooled SP samples. The amounts (μ g) of Td and Pg in each SP sample were then measured by comparisons against whole-cell standards of these micro-organisms, as determined by the ELISA. A biotin-avidin system was used to enhance the sensitivity of the ELISA.

Statistical analyses

The Pearson Product Point Correlation Coefficient was used for the analysis of

site-specific data. Spearman Rank Order of Means correlations were determined for inter- and intra-patient analysis and mean r values were tested for statistical significance. A χ^2 test was used to evaluate synergism between Pg and Td.

Results

Table 1 presents data on SP samples from the group A subjects, representing each of the three ADA case types. The findings, arranged in order for increasing PD values, show a trend toward

higher clinical indices and Pg levels with progression through the data for the 3 case types. However, Td levels show little change, so that the Pg:Td ratio increases markedly with the progression from types I through III.

On the other hand, the TL activities for these subjects do not show much difference according to case type. This may be due in part to the use of smaller SP samples than were tested with the group B subjects. The variability among subjects within the case types can also be due to the very low levels of Td and

Pg found for certain individuals or to the presence of other microorganisms, such as *Bacteroides forsythus*, *B. denticola*, or certain *Capnocytophaga* sp. (e.g., *C. sputigena*), that can elaborate TL activity (Seida et al. 1992, Pederson & Lamberts 1990). For example, subject #9 of the case type II set had neither Pg nor Td at levels detectable by the ELISA; however, rather high levels of TL activity were found. On the other hand, Subject #3 of the type III set, with a very low Pg level and no detectable Td, showed no TL activity.

Table 1. Group A subjects. Relationships among clinical indices and levels of *P. gingivalis* and *T. denticola* in SP samples, and TL activity (mean data from sites sampled for individual subjects)

ADA case type	Subject	N ⁺	Clinical indices		SP samples			TL activities (intervals (min))				
			GI	PD	$\mu\text{g Pg}$ mg SP	$\mu\text{g Td}$ mg SP	Ratio Pg:Td	5	10	15	30	60
I	1	6	0.0	2.3	1.4	1.6	0.9	0.00	0.00	0.17	0.25	0.58
	2	8	0.0	2.5	2.0	2.1	1.0	0.00	0.00	0.06	0.06	0.19
	3	8	0.4	2.5	1.9	1.6	1.2	0.00	0.00	0.00	0.06	0.13
	4	8	0.0	2.6	2.5	2.2	1.2	0.00	0.00	0.00	0.06	0.31
	5	8	0.0	2.9	2.2	1.9	1.2	0.00	0.06	0.06	0.25	0.63
	6	7	0.1	2.9	0.5	1.3	0.4	0.00	0.07	0.14	0.36	0.64
	7	8	0.1	2.9	2.3	2.1	1.1	0.00	0.00	0.00	0.00	0.06
	8	4	0.0	3.0	2.7	1.2	2.2	0.00	0.00	0.00	0.00	0.00
mean* (\pm sd)			0.1	2.7	1.9	1.8	1.2	0.00	0.02	0.05	0.13	0.32
			(0.1)	(0.3)	(0.7)	(0.4)	(0.5)	(0.00)	(0.03)	(0.07)	(0.14)	(0.26)
II	1	8	0.0	2.3	0.0	0.0	—	0.06	0.13	0.13	0.13	0.13
	2	12	0.3	2.4	15.0	1.2	12.5	0.00	0.19	0.25	0.35	0.58
	3	8	0.4	2.5	7.0	0.5	14.0	0.06	0.19	0.25	0.44	0.44
	4	8	0.4	2.5	12.6	3.2	3.9	0.00	0.00	0.00	0.06	0.13
	5	8	0.5	2.5	11.6	2.9	4.0	0.13	0.22	0.22	0.25	0.31
	6	8	0.0	2.6	6.2	0.5	12.4	0.06	0.09	0.13	0.13	0.25
	7	8	0.0	2.8	25.5	0.0	—	0.06	0.06	0.06	0.19	0.81
	8	8	0.1	2.8	1.2	0.0	—	0.00	0.06	0.13	0.13	0.13
	9	8	0.3	2.8	0.0	0.0	—	0.19	0.25	0.31	0.31	0.69
	10	8	0.0	3.0	0.5	1.1	0.5	0.00	0.06	0.06	0.13	0.25
	11	8	0.1	3.0	2.9	2.9	1.0	0.00	0.13	0.31	0.31	0.81
	12	8	1.6	3.3	9.9	3.5	2.8	0.19	0.31	0.47	0.84	0.94
mean* (\pm sd)			0.3	2.7	7.7	1.3	6.4	0.06	0.14	0.19	0.27	0.46
			(0.4)	(0.3)	(7.7)	(1.4)	(5.6)	(0.07)	(0.09)	(0.13)	(0.21)	(0.30)
III	1	6	0.0	2.5	14.9	4.2	3.5	0.08	0.08	0.08	0.17	0.42
	2	8	0.4	3.0	15.6	3.9	4.0	0.06	0.06	0.13	0.19	0.31
	3	8	0.1	3.1	2.5	0.0	—	0.00	0.00	0.00	0.00	0.00
	4	8	0.4	3.3	6.4	1.7	3.8	0.00	0.06	0.06	0.34	0.50
	5	8	0.4	3.4	27.7	3.5	7.9	0.00	0.06	0.06	0.06	0.19
	6	8	0.4	3.5	23.9	1.3	18.4	0.00	0.13	0.13	0.13	0.38
	7	8	0.4	3.6	26.7	0.5	53.4	0.00	0.06	0.06	0.06	0.19
	8	8	0.8	3.6	28.0	1.0	28.0	0.13	0.19	0.25	0.34	0.34
	9	8	0.8	3.6	19.2	1.9	10.1	0.13	0.13	0.19	0.31	0.44
	10	8	0.5	3.8	26.0	0.4	65.0	0.19	0.19	0.19	0.19	0.31
	11	8	0.5	3.8	11.5	1.1	10.5	0.06	0.13	0.13	0.13	0.25
	12	8	0.8	3.8	30.3	4.6	6.6	0.06	0.06	0.19	0.19	0.31
	13	8	0.4	4.1	9.4	1.3	7.2	0.00	0.00	0.13	0.22	0.22
	14	8	1.5	4.9	2.9	1.6	1.8	0.00	0.06	0.06	0.19	0.38
	15	8	0.0	5.6	16.2	3.8	4.3	0.00	0.19	0.19	0.25	0.66
	16	8	1.0	6.0	33.6	4.3	7.8	0.00	0.00	0.13	0.25	0.56
mean* (\pm sd)			0.5	3.9	18.4	2.2	15.5	0.04	0.09	0.12	0.19	0.34
			(0.4)	(0.9)	(10.0)	(1.6)	(19.1)	(0.06)	(0.07)	(0.07)	(0.10)	(0.24)

N⁺: no. sites sampled per subject. * mean of subject means.

Table 2. Mean within-subject Spearman rank order correlation of SP and total Td + Pg at 5 incubation periods for group A subjects

Incubation period (min)	T value	Mean within-patient r value	p value
5	-0.47	-0.0500	NS (0.66)
10	1.52	0.1001	NS (0.14)
15	2.40	0.1430	0.0240
30	2.66	0.1675	0.0126
60	4.30	0.2649	0.0002

The TL activities of the SP samples from all group A subjects significantly correlated with PD ($p < 0.003$) after a 30-min incubation. When analyzing site-specific data (Pearson), significant positive correlations were found after a 60-min incubation as follows: TL activity versus $\mu\text{g Td}/\text{mg SP}$ ($p < 0.030$), TL activity versus total $\mu\text{g Td} + \text{Pg}/\text{mg SP}$ ($p < 0.050$) and TL activity versus PD ($p < 0.003$).

Although 46 of the 57 samples from case type I subjects showed no TL activity after 30-min incubations, 11 (20%) of the samples did show activity. The detection of TL activity in SP from apparently clinically healthy sites has also been reported by Loesche et al. (1990b) when utilizing the synthetic substrate N-benzoyl-DL-arginine-2-naphthylamide (BANA).

A determination of TL activity increase with extended incubation time for SP samples (100 ADA type II and 126 type III) showed the following: type II samples increased from 28% to 34% positive after 30 and 60 min respectively, while type III samples increased from 32% to 43% positive after 30 and 60 min respectively.

ELISA data on the SP samples from Group A subjects generally demonstrated higher numbers of both Td and Pg for the case type III than for the case type II subjects. Mean ($\pm \text{sd}$) Td levels of 0.86×10^6 ($\pm 0.64 \times 10^6$) (range 0.00 to 1.82×10^6) were found for the type III subjects compared to 0.44×10^6 ($\pm 0.57 \times 10^6$) (range 0.00 to 1.53×10^6) for the type II subjects. Also, mean ($\pm \text{sd}$) Pg levels of 22.87×10^6 ($\pm 13.00 \times 10^6$) (range 3.62×10^6 to 47.96×10^6) were found for the type III subjects compared to 9.00×10^6 ($\pm 8.02 \times 10^6$) (range 0.00 to 23.27×10^6) for the type II subjects. The difference in Pg levels for the two sets of subjects was significant at the $p < 0.005$ level by the BMDP t-test when evaluated as mean pooled data and at $p < 0.003$ when the data points were compared by subject.

2 other significant correlations were found when the data for the sum of $\mu\text{g Td}$ and $\mu\text{g Pg}$ per mg SP were analyzed by specific site. These were correlations respectively of total $\mu\text{g Td} + \text{Pg}/\text{mg SP}$ versus PD ($p < 0.001$) and versus GI ($p < 0.001$).

Spearman rank order correlations were also computed between SP and total Td + Pg for each subject. One-sample *t*-tests were then used to test whether mean *r* values differed from zero. Table 2 shows the increasing significance between TL-activity level and the total $\mu\text{g Td} + \text{Pg}/\text{mg SP}$ from group A subjects with longer periods of incubation. The TL activity data from the shorter 5-, 10- and 15-min incubations were also analyzed statistically to determine whether significant correlations could be identified, even though only half of each SP collection was used for TL activity assay. Mean correlations differed from zero for total $\mu\text{g Td} + \text{Pg}/\text{mg SP}$ versus GI ($p < 0.01$) and PD ($p < 0.001$) respectively.

The detection frequency data between the levels of Pg and Td in 283 SP samples are shown in Table 3 and demonstrate the synergistic relationship between these micro-organisms in a continental US population. These data confirm the findings of Simonson et al. (1992b), who first identified the synergistic relationship between these putative oral pathogens in three multinational military populations encamped as Sinai Desert observers.

Table 4 presents data on the SP samples from the 10 group B subjects. The table, arranged in order of increasing PD, demonstrates a general trend toward earlier detectable levels of TL activity as the PD increases. Analyses on a site-specific basis for these subjects showed statistically significant correlations between PD and TL activity, at the $p < 0.05$ level for the 5-, 10- and 15-min intervals and at the $p < 0.01$ level following the 30- and 60-min incubations.

Table 5 presents the distribution of TL activity in relation to PD for 93 SP samples from the group B subjects. The number of SP samples showing activity increased with length of incubation period. There were significant positive correlations when the TL activity data were compared to PD at the 5-, 10- and 15-min incubations ($p < 0.030$) and at the 30- and 60-min incubations ($p < 0.001$) using the Spearman *r* values. It required at least a 15-min incubation to achieve a TL-activity index of 3.0 (1 of 93 samples).

Table 6 presents a comparison of PD vs TL activity data, analyzed on a site-specific and by-patient basis respectively. The Pearson *r* values steadily increased with incubation time (10–60 min) and the corresponding *p*-values gained significance (0.0289 at 10 min to 0.0023 at 60 min). The Spearman *r*-values also steadily increased with incubation time (5–60 min) and the corresponding *p*-values gained significance (0.0223 at 5 min to 0.0004 at 30- and 60-min). This suggests an optimal incubation period range of 15–30 min when using the AFC disc for the detection of TL activity in SP samples.

Tests were conducted to determine the mean cell quantities of Td and Pg necessary to create visually detectable reactions with AFC discs after 5 min incubations. Results of tests with micro-organisms cultured 3, 4, and 5 days showed 7.566×10^8 Td cells (range 0.004 – 8.360×10^8 , $n=9$) and 1.307×10^8 Pg cells (range 0.158 – 15.600×10^8 , $n=14$) were needed, regardless of cell viability.

Calculations based on the ELISA data for $\mu\text{g Td}/\text{mg SP}$ and $\mu\text{g Pg}/\text{mg SP}$ (where only one of the two micro-organism types was detectable) indicated that 4.78×10^5 Td cells and 1.36×10^7 Pg cells were needed to detect TL activity in 5 min or less. These numbers are smaller than those shown above for the cultured microorganisms and, as noted earlier, they include both viable and non-viable cells.

The activity of the bovine pancreatic

Table 3. Frequency of microbial detection in 283 SP samples

Detection of <i>T. denticola</i>	Detection of <i>P. gingivalis</i>	
	+	-
+	134	7
-	43	99

$\chi^2 = 135.1$, 1 df, $p < 0.00001$.

Table 4. Group B subjects: relationships of clinical indices with TL activities

ADA case type	Subject	N ⁺	Clinical indices			TL activities (intervals (min))				
			PI	BP*	PD	5	10	15	30	60
III	1	8	0.8	0.3	3.4	0.19	0.31	0.44	0.72	0.88
	2	15	1.7	0.2	3.6	0.00	0.07	0.17	0.30	0.40
	3	12	1.8	0.3	3.7	0.08	0.17	0.25	0.29	0.46
	4	8	2.1	0.1	4.1	0.00	0.00	0.00	0.19	0.44
	5	7	1.4	0.4	4.4	0.00	0.21	0.21	0.89	1.04
	6	8	0.6	0.0	5.3	0.19	0.50	0.63	1.13	1.34
mean** (+sd)			1.4 (0.6)	0.2 (0.1)	4.1 (0.7)	0.08 (0.09)	0.21 (0.18)	0.28 (0.22)	0.59 (0.38)	0.76 (0.39)
IV	1	8	1.6	0.4	5.8	0.13	0.38	0.56	1.19	1.63
	2	16	2.3	0.4	5.8	0.19	0.28	0.34	0.66	0.88
	3	4	2.0	0.3	5.8	0.50	0.81	0.88	1.19	1.88
	4	4	3.0	1.0	5.8	0.25	0.38	0.63	1.00	1.38
	mean** (±sd)		2.2 (0.6)	0.5 (0.3)	5.8 (0.0)	0.27 (0.16)	0.46 (0.24)	0.60 (0.22)	1.01 (0.25)	1.44 (0.43)

N⁺: no. sites sampled per subject.

* Bleeding on probing, 0=no and 1=yes.

** Mean of subject means.

Table 5. Relationship of TL activity distribution to mean probing depth (mm) for group B samples over 5 incubation periods

TL-activity rating	Incubation period				
	5 min*	10 min*	15 min*	30 min**	60 min**
0.0	77/4.6	67/4.6	58/4.5	49/4.3	40/4.2
0.5	7/5.0	9/4.7	15/5.2	9/5.0	10/4.9
1.0	9/5.7	10/5.3	9/5.0	14/5.5	16/5.3
1.5	0/-	2/6.0	5/5.2	6/5.8	4/6.0
2.0	0/-	5/5.2	5/5.8	11/4.6	16/5.3
2.5	0/-	0/-	0/-	1/7.0	0/-
3.0	0/-	0/-	1/4.0	3/5.3	7/5.1
total samples	93	93	93	93	93

* Significant at $p < 0.030$, Spearman.** Significant at $p < 0.001$, Spearman.Table 6. Relationship of r and p values over time for PD versus TL-activity levels

Incubation period (min)	Pearson	Pearson	Spearman	Spearman
	r	p	r	p
5	0.2502	0.0167	0.2407	0.0223
10	0.2292	0.0289	0.2736	0.0091
15	0.2335	0.0259	0.3172	0.0023
30	0.2911	0.0051	0.3629	0.0004
60	0.3154	0.0023	0.3648	0.0004

trypsin could be detected within 5 min when 5 pg were applied to AFC discs in the collection buffer. However, 10 to 25 pg produced a much stronger response within the same period. When the same trypsin standards were applied to the non-fluorescing synthetic substrate N- α -benzoyl-DL-arginine-p-nitroanilide (BAPNA) in the agar-gel format of Pederson & Lamberts (1990), substantial differences between the sensitivity of the two substrates were observed. The BAPNA system produced a

visually positive response with 500 to 1000 ng of the bovine pancreatic trypsin in about 30 min. Therefore, TL activity detection with the AFC disc may be as much as 20 to 200 times more sensitive than the BAPNA gel method and may yield results in 1/6 the time.

Discussion

Loesche et al. (1987) employed BANA to demonstrate the TL-activity of SP containing high proportions of spiro-

chetes, and reported a significant association between BANA hydrolysis and increased PD. Our results confirm the importance of Td and Pg levels in SP for the clinical assessment of periodontal health and provide additional correlations between the levels of these microorganisms in SP and various clinical parameters. The presence in SP of other TL activity-producing micro-organisms besides Td and Pg has been demonstrated by Seida et al. (1992) with the SK-013 enzymatic detection method. Such micro-organisms may have contributed to the TL activity of our SP samples when Td or Pg levels were extremely low or could not be detected by our methodology.

The present report is the first to identify the correlation between AFC fluorescence and the presence of Td and Pg in SP, as determined by highly specific monoclonal antibodies to these micro-organisms. It also first identifies the synergistic relationship between Pg and Td in a continental US American population and confirms the findings of Simonson et al. (1992a & 1992b).

In the tests with group A subjects, 15-min incubations with half of routine single-pass SP collections were found sufficient to provide a significant positive correlation of AFC fluorescence with total Td+Pg levels. This observation suggested that the SP samples collected in single currettings might be adequate to yield a similar correlation within 5–10 min. The group B data demonstrated that this was indeed the case. Statistically significant correlations were found after only a 5-min incubation, with higher degrees of significance readily achievable by extending the incubation period. However, longitudinal studies are required to determine whether the degree of fluorescence can be significantly correlated with changes in clinical parameters.

We have approximated the levels of viable and non-viable Td and Pg cells necessary to yield a positive response in 5 min, both by cell culture and by monoclonal antibody methodologies. Further studies are required to determine the threshold levels of detectability of TL-activity-producing micro-organisms by AFC discs.

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Zusammenfassung

Höhe der Aktivität von Trypsin-ähnlichen Enzymen von Treponema denticola und Porphyromonas gingivalis bei Erwachsenen mit Parodontitis

Treponema denticola (Td) und *Porphyromonas gingivalis* (Pg) werden mit moderater bis schwerer Erwachsenenparodontitis assoziiert. In dieser Studie wurden diese zwei Anaerobier und ihre Aktivität von Trypsin-ähnlichen Enzymen (TL) in subgingivaler Plaque gemessen, die von klinisch gesunden und parodontal erkrankten Stellen von Patienten mit Parodontitis entnommen wurde. Die Antigenmenge der Mikroorganismen wurde mit monoklonalen Antikörpern bestimmt und die TL-Aktivitäten über ein scheibenförmiges Z-gly-gly-arg-AFC-Substrat gemessen. Die statistische Analyse der Daten zeigte sowohl bei flächenspezifischer als auch patientenspezifischer Auswertung eine signifikant positive Korrelation zwischen der Antigenmenge und den TL-Aktivitäten. Bei einer kontinentalen US-Population wurde ein anaerober Synergismus zwischen Td und Pg gefunden, sowie eine positive Korrelation zwischen der Menge der Anaerobier (individuell und insgesamt) und den klinischen Befunden der Erwachsenenparodontitis.

Résumé

Niveaux d'activité semblable à la trypsine de Treponema denticola et de Porphyromonas gingivalis chez les adultes atteints de parodontite

Treponema denticola (Td) et *Phorphyromonas gingivalis* (Pg) sont associés à la maladie parodontale modérée et sévère de l'adulte chez l'humain. Le présent travail est une étude quantitative de ces deux anaérobies et de leurs activités de type trypsine (TL) dans la plaque sous-gingivale recueillie au niveau de sites cliniquement sains et de sites avec pathologie parodontale chez des humains atteints de parodontite. Les niveaux d'antigène dans les microorganismes ont été déterminés à l'aide d'anticorps monoclonaux et les activités TL ont été mesurées avec le substrat fluorescent Z-gly-gly-arg-AFC en disques. Des corrélations positives significatives ont été

observées entre les niveaux d'antigène et les activités TL quand l'analyse statistique des données était pratiquée tant sur une base spécifique du site qu'en se basant sur le patient. Un synergisme anaérobie a été trouvé entre Td et Pg dans une population continentale des États-Unis, et des corrélations positives ont été trouvées entre les niveaux d'anaérobies (individuellement et au total) et les indicateurs cliniques de la parodontite de l'adulte.

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Bactericidal Activity of PMN Factors on *Treponema denticola*. E. D. PEDERSON*,
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Current evidence indicates that polymorphonuclear neutrophils [PMNs] and other phagocytes play a major role in controlling microorganisms in the periodontium [Miyasaki, 1991], thereby protecting the host against periodontal pathogens and diseases. Levels of the pathogenic oral spirochete *Treponema denticola* [Td] have been shown to increase as periodontal pocket depth increases [Simonson, 1988]. The aim of this study was to determine whether bactericidal actions of certain PMN factors and Td could be detected *in vitro*. Four purified PMN factors, elastase [PMNE], myeloperoxidase [MPO], lysozyme [LYSO] and cathepsin-G [Cath-G] were tested on Td strains ATCC 35405 and VPI D39DP1. Bacterial cell concentrations, determined spectrophotometrically at 660 nm, were $1-5 \times 10^7$ Td cells/ml. Td and PMN factors were placed in saline containing 20 mM MgCl₂ for testing. Treatments consisted of combining 50 µl of each Td suspension [0.5-2.5 $\times 10^6$ cells], and 0.23, 1.39 or 8.33 µg of each factor [50 µl] in duplicate wells of low background fluorescence 96-well microtiter plates. Live/Dead^R BacLight Viability dyes and a Fluoroskan II microplate reader were employed to distinguish live from dead [L vs D] Td. L/D ratios of three replicate readings made at 30 min intervals, compared to controls, were used to determine Td killing. Of the four PMN factors tested, only Cath-G demonstrated detectable microbicidal activity [$p < 0.01$ to $p < 0.001$] for the two Td isolates when compared to PMNE, MPO and LYSO, at the 0.23, 1.39 and 8.33 µg levels. The results indicate that Td is more sensitive to killing by cathepsin-G than by the other factors examined in this pilot study. Results were verified by the DAPI/PI method of Turner, 1995. Supported by the Office of Naval Research, Naval Medical Research and Development Command, Bethesda, Maryland, project number 0601152N MR00001.001-0064.

"Aerobic" Cultivation of *Porphyromonas gingivalis*. E.D. PEDERSON*, D.W. TURNER, C. MERRITT, C.T. GLYNN and L.G. SIMONSON [The Naval Dental Research Institute, Great Lakes, Illinois, 60088, USA].

Porphyromonas gingivalis [Pg], a microorganism frequently associated with human periodontal diseases, is a strict anaerobe. The aim of this study was to develop a simple method for cultivating Pg under "aerobic" conditions while not requiring any specialized equipment. Wilkins-Chalgren [W-C] broth was supplemented with 20 ml/L 10% sodium bicarbonate [SB], cysteine hydrochloride [C] and sodium thioglycolate [ST] as reducing agents, and 5 mg/L additional hemin [H] and 50 µg/L menadione [M]. Growth requirements for SB, C, ST, H, and M by various Pg isolates were determined over 48-to-72-hour incubations at 37°C under aerobic conditions. Only those cultures receiving SB, C, and ST displayed growth, and the addition of H & M further enhanced early growth rates. Optimal levels of the required agents were found to be 10 ml/L of 10% SB, 0.50 g/L of C, and 0.25 g/L of ST. Nine Pg isolates from the Virginia Polytechnic Institute and University, the University of Michigan and the American Type Culture Collection were cultured concurrently, using the optimal SB, C, and ST levels without added H & M. Cell numbers of Pg isolates doubled [D86B6, JKG-7, 33277], tripled [D13B11, D67D9, D84D2] or sextupled [JKG-1, D82F5] over the first 24 hours of incubation. This modification of W-C broth has provided an excellent medium for the growth of oral Pg under "aerobic" conditions, and simplifies handling procedures to facilitate studies at the bench top or in biologic cabinets. Supported by the Office of Naval Research, the Naval Medical Research and Development Command, Bethesda, Maryland, Project Number 0601152N MR00001.001-0064.

Fluorescence Stain for Estimating *Treponema denticola* Viability.
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Treponema denticola [Td] has been found to increase in subgingival plaque in direct proportion to periodontal disease [Simonson, 1988]. Because of the difficulty in cultivation of spirochetes, a method that results in quantitative viable counts in a convenient, reliable and reproducible manner is desirable for investigations of these microorganisms. The aim of this study was to test a combination of two fluorochromes [Cutler, 1991] for detecting live and dead Td in liquid medium. American Type Culture Collection Td strain 35405 was grown in 1186 medium with 10% inactivated fetal calf serum and 10% NaHCO₃ for 4-8 days in an anaerobic chamber at 37°C with 85% N₂, 5% H₂ and 10% CO₂. Td was pelleted and resuspended in spirolate [SP] broth at OD 0.020, using a Beckman DU 65 spectrophotometer at 660 nm. One ml of the Td suspension was labeled with 7.5 µg 4',6-diamidino-2-phenylindole for 10 minutes at room temp. The microorganisms were washed twice with 1 ml and resuspended in 0.5 ml SP broth, and propidium iodide was added at a final concentration of 5 µg/ml. Sixty-µl volumes were removed and added to sample chambers on a Cytospin apparatus and centrifuged at 850 rpm for 3 min. Dried samples were fixed under a coverslip and examined on a Zeiss Photomicroscope II equipped with a 405 nm excitation filter and a 475 nm barrier filter. The method was found to be satisfactory for differentiating living [blue] from dead [red] Td cells. This study was supported by the Office of Naval Research, the Naval Medical Research and Development Command, Bethesda, Maryland, Project Number 0601152N MR00001.001-0064.